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THE INFLUENCE OF CHEMICAL CONSTITUTION ON ANTIBACTERIAL ACTIVITY. PART IV: A SURVEY OF HETEROCYCLIC BASES, WITH SPECIAL REFERENCE TO BENZQUINOLINES, PHENANTHRIDINES, BENZACRIDINES, QUINOLINES AND PYRIDINES.

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A CLOSE correlation between kationic ionization and antibacterial properties has been demonstrated in the acridine series (Albert, Rubbo, Goldacre, Davey and Stone, 1945; Albert and Goldacre, 1948). Those acridines which are sufficiently strong bases to be more than 50 per cent ionized at pH 7·3 were found to exert a strong bacteriostatic action, whereas weaker bases were found relatively ineffective. Because no other series of bases has ever been investigated along these lines, it was not known whether this type of correlation pertains only to the acridine series or is capable of wider application.

The aim of the present work has been to extend this investigation to a representative number of other heterocyclic series in order to discover whether these would resemble the acridines in that the highly ionized members would be more antibacterial than the poorly ionized members.

The results of these investigations plainly showed that kationic ionization markedly increases antibacterial action in the following seven series, the three isomeric benzquinoline series, the phenanthridines, and the three isomeric benzacridine series.

All these substances have molecules with a large area of flat surface—an architectural feature which was shown to be necessary for this type of antibacterial action. When it was incorporated in the quinoline and the pyridine series, the correlation between ionization and antibacterial properties was again easily demonstrated. These results led to a general discussion of the factors which govern the antibacterial action of organic bases.

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MATERIALS AND METHODS.

Chemical.

Organic chemistry.

Of the 96 substances listed in Tables I, II, III, IV, V and VII, 33 were previously unknown, and had to be specially synthesized to fill important gaps in the data. The preparation of the new benzquinolines, phenanthridines and benzacridines is described by Albert, Brown and Duewell (1948), and of the older examples by Albert, Goldacre and Phillips (1948). The three series of benzquinolines (II, III and IV; Table I), the phenanthridines (V) and the three series of benzacridines (VI, VII and VIII; Table II) have been numbered according to the British system; those who use the American system will find a key for inter-conversion in Table VIII.

The preparation of the styryl compounds, the guanidines and the diguanides has been described by Royer (1949). The acridine derivatives were described in our previous paper (Albert *et al.*, 1945). The aliphatic amines (No. 87–89) in Table VII were kindly supplied by Dr. K. Sutherland, of the Division of Chemistry, Council for Scientific and Industrial Research, Melbourne. The source of the remainder of the substances is as given by Albert, Goldacre and Phillips (1948), except where otherwise indicated.

Careful attention was paid to bringing all test-substances to a state of complete analytical purity.

Physical chemistry.

The majority of the pK_a values given in this paper have been taken from Albert, Goldacre and Phillips (1948). The others, unless otherwise mentioned in footnotes to the tables, were kindly determined by Mr. J. N. Phillips.

Some of the published pK_a values have been determined only in dilute alcohol, so that it was necessary to add a correction to convert these figures to the equivalent values for water. For each series the magnitude has been obtained by averaging those corrections found necessary where pK_a values have been determined both in water and alcohol, as follows:

Benzquinolines and phenanthridines .	1·0 unit.
Benzacridines	0·7 ,,
Acridines, quinolines and pyridines .	0·5 ,,

Those values which have been obtained by correction have been placed in parentheses in the tables. They are subject to an error of about $\pm$ 0·2 unit, which is of no consequence in the present work except for pK_a values lying between 6·3 and 8·3, at pH 7·3, where a small change in pK_a can cause a relatively large change in percentage ionization because of the shape of the curve at this point. Fortunately, very few values falling within this range had to be corrected.

Bacteriological.

The technique, the test organisms and the medium (Wright's heart broth with added peptone and 10 per cent of sterile, unheated ox serum) were as defined by Albert *et al.* (1945). The usual heavy inoculum was used, viz. 0·03 ml. of a 24-hour culture in 2·5 ml. of medium, thus providing at least one million organisms per tube.

The biological results in the tables are recorded in terms of the actual chemical *base*, in order to eliminate errors due (i) to varying amounts of water of crystallization and (ii) to acid radicals of different molecular weight. The free bases were dissolved in water with the aid of 1 to 1·5 equivalents of hydrochloric or acetic acid.

That dilution completely preventing visible growth after 48 hours' incubation at 37° C. was taken as the end-point of bacteriostasis. To this dilution a code number was assigned according to the following key :

Key to dilutions to be used for all tables dealing with antibacterial activity.

0	signifies	growth at	1 in	5000	(calculated as base).
1	,,	inhibition of growth at	1 in	5000	
2	,,	,,	,,	1 ,,	10,000
3	,,	,,	,,	1 ,,	20,000
4	,,	,,	,,	1 ,,	40,000
5	,,	,,	,,	1 ,,	80,000
6	,,	,,	,,	1 ,,	160,000
7	,,	,,	,,	1 ,,	320,000
8	,,	,,	,,	1 ,,	640,000

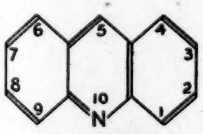
It will be appreciated that, as these figures vary by a factor of two, a substance giving the reading "6" is eight times (2^3) as powerful as one giving the reading "3."

All tests were done in duplicate. In the case of all substances having special bearing on the hypotheses developed here, the tests were repeated, again in duplicate.

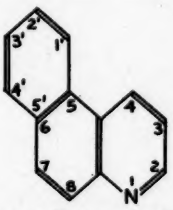
RESULTS.

(i) *The Isomerides of Acridine.*

Acridine may be regarded as 2:3-benzquinoline. It was therefore decided to examine all the isomerides arising when one benzene ring in acridine is moved

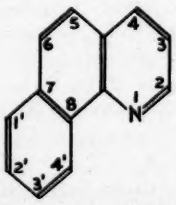


(I, Acridine)



(II, 5:6-Benzquinoline)

(III, 6:7-Benzquinoline)



(IV, 7:8-Benzquinoline)

(V, Phenanthridine)

TABLE I.—*Antibacterial Properties of Benzquinolines and Phenanthridines. Highest Dilutions Completely Preventing Visible Growth in 48 hours at 37° C.*

Medium: 10 per cent serum broth, pH 7·2–7·4.

No.	Substance.	Organisms.					Bacteriostatic index (sum total of code numbers of inhibitory dilutions).	pKa in water at 20° C.	Per cent ionized (kation) at 20° C., pH 7·3.
		Cl. welchii.	*Str. pyogenes.*	*Staph. aureus.*	*B. coli.*	*Proteus.*			
1.	5-*Aminoacridine* (for comparison)	6	6	4	4	4	24	10·0	100
	5:6-*Benzquinoline*—								
2.	(Unsubstituted)	1	1	1	0	0	3	5·1	<1
3.	1′-Amino-	2	0	0	0	0	2	5·0	<1
4.	3′-Amino-	0	0	0	0	0	0	(5·0)	<1
5.	4′-Amino-	1	1	0	0	0	2	5·2	<1
6.	2-Methyl-	2	1	1	0	0	4	(5·4)	1
7.	2′:4′-Diamino-	0	0	0	0	0	0	(5·9)	4
8.	2-Amino-4-methyl-	4	3	0	0	0	7	7·1	39
9.	4-Amino-	6	3	2	3	2	16	(9·0)	98
10.	4-Amino-2-methyl-	5	4	2	3	1	15	(9·4)	99
	6:7-*Benzquinoline*—								
11.	(Unsubstituted)	1	2	0	0	0	3	5·0	<1
12.	8-Chloro-	0	0	0	0	0	0	(3·5)	<1
13.	4-Amino-2-methyl 8-chloro-	2	2	0	0	0	4	(6·9)	28
14.	3:4-Diamino-	5	4	2	2	0	13	(9·1)	98
15.	4-Amino-	7	4	3	3	2	19	(9·7)	100
16.	4-Amino-2-methyl-	6	5	3	3	1	18	(10·4)	100
	7:8-*Benzquinoline*—								
17.	(Unsubstituted)	2	0	0	0	0	2	4·2	<1
18.	1′-Amino-2-methyl-	0	0	0	0	0	0	(5·7)	3
19.	6-Amino-2-methyl-	0	0	0	0	0	0	(6·2)	7
20.	2-Amino-4-methyl-	2	0	0	0	0	2	6·7	20
21.	4-Amino-	5	5	3	4	2	19	(8·7)	96
22.	4-Amino-2-methyl-	6	5	3	3	1	18	(9·0)	98
	Phenanthridine—								
23.	(Unsubstituted)	2	2	1	0	0	5	(4·3)	<1
24.	7-Amino-9-methyl-	0	0	0	0	0	0	(6·2)	7
25.	2-Amino-9-methyl-	3	2	1	0	0	6	(6·7)	20
26.	9-Amino-	4	4	2	2	2	14	7·3	50
27.	2:7-Diamino-9-methyl-	5	4	3	1	0	13	(7·3)	50
28.	2:7:9-Triamino-	6	5	4	1	0	16	(9·1)	98

to all the other possible positions on the quinoline skeleton. Thus 3:4-benzquinoline (which is known as "phenanthridine"), and the 5:6-, 6:7- and 7:8-benzquinolines, which do not have special names, were examined. To these were added a sufficient number of their amino-derivatives, so chosen as to cover the range from the almost non-ionized to the almost completely ionized at the pH of the test, viz. pH 7·3. The results are given in Table I, and the relevant structural formulae are at the head of this table. 5-Aminoacridine (Aminacrine, B.P.), which is one of the best of the highly ionized acridines, has been included as a standard.

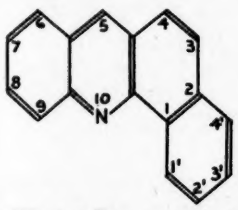
It is seen from Table I that a strong positive correlation exists, in all four series, between the ionization of the bases and their antibacterial action. As

soon as the basic strength is high enough to bring about ionization to the extent of 50 per cent or more, the antibacterial titre increases considerably. In short, the bacteriostatic index remains below 7 as long as the ionization remains below 50 per cent ; but when the ionization equals or exceeds this figure, scores as high as 19 (e.g. 4-amino-7:8-benzquinoline, No. 21) are obtained.

Although this correlation resembles that found in the acridine series, it is evident that the highest score, 19, falls short of the figure, 24, obtained with 5-aminoacridine (No. 1), and that the four series exemplified in Table I are not all equally active.

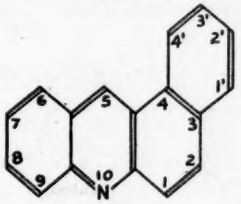
(ii) *The Benzologues of Acridine.*

Benzologues are those substances differing from one another by the presence of more, or fewer, benzene rings, each of which shares two carbon atoms with



(VI, 1:2-Benzacridine)

(VII, 2:3-Benzacridine)



(VIII, 3:4-Benzacridine)

TABLE II.—*Antibacterial Properties of Benzacridines. Highest Dilutions Completely Preventing Visible Growth in 48 hours at 37° C.*

Medium: 10 per cent serum broth, pH 7·2–7·4.

No.	Substance.	Organisms. *Cl. welchii.*	*Str. pyogenes.*	*Staph. aureus.*	*B. coli.*	*Proteus.*	Bacteriostatic index (sum total of code numbers of inhibitory dilutions).	pKa in water at 20° C.	Per cent ionized (kation) at 20° C., pH 7·3.
	1:2-*Benzacridine*—								
29.	(Unsubstituted)	0	0	0	0	0	0	(4·1)	<1
30.	7-Amino-	0	0	0	0	0	0	(4·7)	<1
31.	8-Amino-	4	4	3	0	0	11	6·7	20
32.	5-Amino-4′-nitro-	6	1	0	0	0	7	(7·1)	39
33.	5-Amino-	8	6	6	3	0	23	(8·8)	97
34.	4′:5-Diamino-	7	7	6	3	2	25	(9·1)	98
	2:3-*Benzacridine*—								
35.	(Unsubstituted)	1	2	0	0	0	3	(5·2)	<1
36.	5-Acetamido-	6	0	0	0	0	6	(5·3)	1
37.	7-Amino-	2	0	0	0	0	2	(6·1)	6
38.	5-Amino-	7	7	5	4	1	24	(10·4)	100
	3:4-*Benzacridine*—								
39.	(Unsubstituted)	0	0	0	0	0	0	4·7	<1
40.	8-Acetamido-	0	0	0	0	0	0	(5·2)	<1
41.	7-Amino-	0	0	0	0	0	0	(5·7)	3
42.	8-Dimethylamino-	4	5	2	0	0	11	7·3	50
43.	8-Amino-	7	5	5	0	0	17	7·4	56
44.	5-Amino-	7	6	5	3	2	23	(9·1)	98

another ring. The most familiar example of benzologues is the family : benzene, naphthalene and anthracene. Pyridine, quinoline, acridine and the benzacridines form a similar family of benzologues, the members of which have now been examined for evidence of correlation between ionization and antibacterial action (Tables II and III). A sufficient number of amino-derivatives have been included to cover a wide range of percentage ionization, and, in the case of the aminoquinolines and aminopyridines, every possible isomeride has been tested.

It is seen from Table II that a strong positive correlation exists, in all three benzacridine series, between the strength of the bases and their antibacterial action. Those bases strong enough to be more than 50 per cent ionized are very much more antibacterial than those which fall below this figure. The high scores in this series (e.g. a bacteriostatic index of 23 to 25 for No. 33, 34, 38 and 44) show it to be the equal of the acridine series (*cf.* 5-aminoacridine, No. 1, one of the best of the amino-acridines).

(IX, Quinoline)

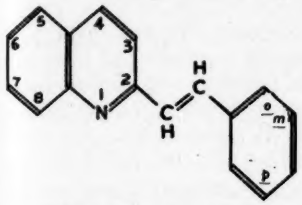
(X, Pyridine)

TABLE III.—*Antibacterial Properties of Quinolines and Pyridines. Highest Dilutions Completely Preventing Visible Growth in 48 hours at 37° C.*

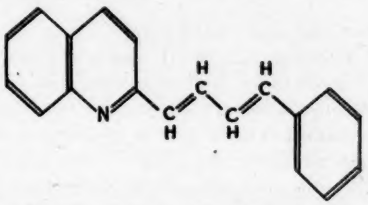
Medium: 10 per cent serum broth, pH 7·2–7·4.

No.	Substance.	Organisms.					Bacteriostatic index (sum total of code numbers of inhibitory dilutions).	pKa in water at 20° C.	Per cent ionized (kation) at 20° C., pH 7·3.
		Cl. welchii.	*Str. pyogenes.*	*Staph. aureus.*	*B. coli.*	*Proteus.*			
	Quinoline—								
45.	(Unsubstituted)	0	0	0	0	0	0	4·9	<1
46.	8-Amino-	0	0	0	0	0	0	4·0	<1
47.	3-Amino-	0	0	0	0	0	0	4·9	<1
48.	5-Amino-	0	0	0	0	0	0	5·5	2
49.	6-Amino-	0	0	0	0	0	0	5·6	2
50.	7-Amino-	0	0	0	0	0	0	6·6	17
51.	2-Amino-	0	0	0	0	0	0	7·3	50
52.	4-Amino-	0	0	0	0	0	0	9·1	98
53.	2:4-Diamino-	2	2	0	0	0	4	9·4	99
54.	4-Amino-2-methyl-	0	0	0	0	0	0	9·4	99
55.	4-Amino-2:6:8-trimethyl-	2	2	0	1	0	5	10·4	100
56.	4-Amino-2-phenyl-	3	2	1	1	0	7	(8·2)	89
	Pyridine—								
57.	(Unsubstituted)	0	0	0	0	0	0	5·2	<1
58.	3-Amino-	0	0	0	0	0	0	6·0	5
59.	2-Amino-	0	0	0	0	0	0	6·9	28
60.	4-Amino-	0	0	0	0	0	0	9·1	98

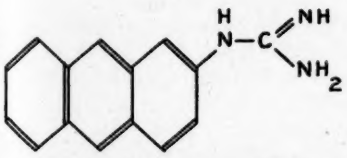
It is seen from Table III that the quinolines and pyridines, which are the lower benzologues of the acridines, are almost devoid of antibacterial action, even when well ionized. Their lack of antibacterial properties can be explained



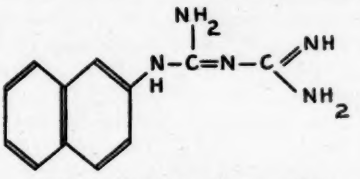
(XI, 2-Styrylquinoline)



(XII, 2-(δ-Phenylbutadienyl)-quinoline)



(XIII, 2-Anthrylguanidine)



(XIV, 2-Naphthyldiguanide)

TABLE IV.—*The Effect upon Antibacterial Action of Decreasing or Increasing the Flat Area of Various Molecules. Highest Dilutions Completely Preventing Visible Growth in 48 hours at 37° C.*

Medium: 10 per cent serum broth, pH 7·2–7·4.

No.	Substance.	Organisms. *Cl. welchii.*	*Str. pyogenes.*	*Staph. aureus.*	*B. coli.*	*Proteus.*	Bacteriostatic index (sum total of code numbers of inhibitory dilutions).	pKa in water at 20° C.	Per cent ionized (kation) at 20° C., pH 7·3.
A.	*Area diminished—*								
61.	5-Amino-6:7:8:9-tetrahydroacridine	0	1	0	0	0	1	(9·9)	100
(1).	(5-Aminoacridine for comparison)	6	6	4	4	4	24	10·0	100
62.	5-Amino-6:7:8:9-tetrahydro-2:3-benzacridine	3	4	4	2	0	13	(10·3)	100
(38).	(5-Amino-2:3-benzacridine for comparison)	7	7	5	4	1	24	(10·4)	100
B.	*Area increased—*								
63.	6-Amino-2-styrylquinoline	0	0	0	0	0	0	(5·5)	2
(49).	(6-Aminoquinoline for comparison)	0	0	0	0	0	0	5·6	2
64.	4-Amino-2-styrylquinoline	5	5	4	2	0	16	(9·3)	99
(52).	(4-Aminoquinoline for comparison)	0	0	0	0	0	0	9·1	98
65.	4-Amino-2-(*p*-aminostyryl) quinoline	5	5	2	3	0	15	*ca.*9·5	99
66.	4-Amino-2-(*p*-dimethylaminostyryl) quinoline	4	4	4	2	0	14	(9·7)	99
67.	4-Amino-6-ethoxy-2-styrylquinoline	5	6	4	0	0	15	*ca.*9·5	99
68.	4-Amino-6-ethoxy-2-(δ-phenylbutadienyl)quinoline (XII)	6	6	3	0	0	15	*ca.*9·5	99
69.	4-Amino-2:6-distyrylpyridine	5	5	3	1	0	14	(8·6)	95
(60).	(4-Aminopyridine for comparison)	0	0	0	0	0	0	9·1	98
C.	*Series of increasing area—*								
70.	Phenylguanidine*	0	0	0	0	0	0	10·9‡	100
71.	2-Naphthylguanidine	0	0	0	0	0	0	10·7	100
72.	2-Anthrylguanidine (XIII)	4	4	4	0	0	12	11·0	100
73.	Phenyl-N^1-diguanide†	0	0	0	0	0	0	10·7	100
74.	2-Naphthyl-N^1-diguanide† (XIV)	1	1	0	0	0	2	10·7	100
75.	2-Anthryl-N^1-diguanide	4	4	4	0	0	12	10·4	100

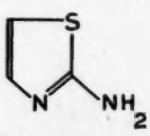
* Prepared as in Smith (1929).
† Prepared as in Cohn (1911) and Smolka and Halla (1901).
‡ From Davis and Elderfield (1932).

by the decreased area of the surface of these molecules. It has been noted (Albert *et al.*, 1945) that a highly significant decrease in antibacterial properties follows the hydrogenation of 5-aminoacridine to 5-aminotetrahydroacridine, an operation which reduces the flat area of the parent molecule. This important phenomenon has been confirmed, as shown in Table IV, where the activities of 5-aminoacridine (No. 1) and 5-aminotetrahydroacridine (No. 61) are compared. An attempt was made to restore antibacterial action to the quinoline, pyridine and tetrahydroacridine series by increasing the area of the flat surface of their molecules in various ways, which will now be described. These modifications of structure will be seen to have caused a profound increase in antibacterial activity.

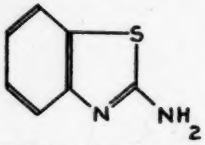
It can be seen from Table IV that the addition of a styryl group to 4-aminoquinoline (No. 52), which is not antibacterial, gives 4-amino-2-styrylquinoline (No. 64), thereby introducing into the quinoline series antibacterial properties of the same order as the best of the benzquinolines. No. 65, 66, 67 and 68 are further examples. Similarly, the addition of two styryl groups to 4-aminopyridine (No. 60), which is not antibacterial, gives 4-amino-2:6-distyrylpyridine (No. 69), which has considerable antibacterial activity. 6-Amino-2-styrylquinoline (No. 63) has been included to demonstrate that an increase in flat surface does not of itself increase antibacterial action : the base must be strong enough to secure a high degree of ionization at the pH of the test.

The addition of a flat ring to 5-aminotetrahydroacridine (No. 61) gave its benzologue, 5-aminotetrahydrobenzacridine (No. 62). Parallel to this restoration of flatness, a considerable proportion of the antibacterial activity is seen to have been restored.

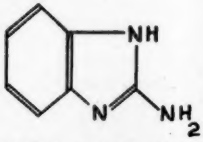
No. 70–75 are two series of non-heterocyclic benzologues, the arylguanidines and the aryl-diguanides. It is seen from Table IV that here also a strong antibacterial action does not appear until a sufficient area of flat surface of the molecule has been built up as in the three-ring anthracene nucleus of No. 72 and 75.



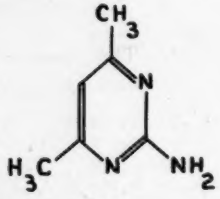
(XV, 2-Aminothiazole)



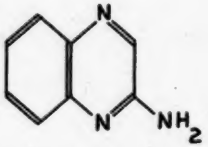
(XVI, 2-Aminobenzthiazole)



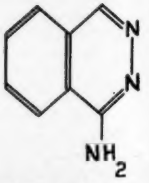
(XVII, 2-Aminobenziminazole)



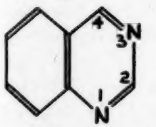
(XVIII, 2-Amino-4:6-dimethylpyrimidine)



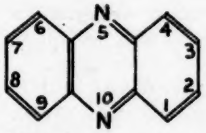
(XIX, 2-Aminoquinoxaline)



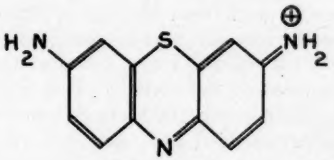
(XX, 1-Aminophthalazine)



(XXI, Quinazoline)



(XXII, Phenazine)



(XXIII, Thionine)

TABLE V.—*Examples of Lack of Antibacterial Activity in Heterocyclic Amines which have One or More of the Following Defects:* (a) *Insufficiently Ionized,* (b) *Insufficient Flat Area per Molecule,* (c) *Well Ionized, but Easily Reduced to Poorly Ionized Substance. Highest Dilutions Completely Preventing Visible Growth in* 48 *hours at* 37° *C.*

Medium: 10 per cent serum broth, pH 7·2–7·4.

No.	Substance.	Type of defect.	Organisms.					Bacteriostatic index (sum total of code numbers of inhibitory dilutions).	pKa in water at 20° C.	Per cent ionized (kation) at 20° C., pH 7·3.
			Cl. welchii.	*Str. pyogenes.*	*Staph. aureus.*	*B. coli.*	*Proteus.*			
76.	2-Aminothiazole (XV)	A, B	0	0	0	0	0	0	5·4	1
77.	2-Aminobenzthiazole (XVI)	A, B	0	0	0	0	0	0	4·5	<1
78.	2-Aminobenziminazole (XVII)	B	0	0	0	0	0	0	7·5	61
79.	2-Amino-4:6-dimethylpyrimidine (XVIII)	A, B	0	0	0	0	0	0	4·8	<1
80.	2-Aminoquinoxaline (XIX)	A, B	0	0	0	0	0	0	4·0	<1
81.	1-Aminophthalazine (XX)	A, B	0	0	0	0	0	0	6·6	17
82.	2-Aminoquinazoline	A, B	0	0	0	0	0	0	4·4	<1
83.	4-Aminoquinazoline	A, B	0	0	0	0	0	0	5·7	3
84.	2-Amino-8-dimethylamino-3-methylphenazine (neutral red)	A	0	0	0	0	0	0	6·9*	28
85.	Thionine (Lauth's violet) (XXIII)	C	2	1	2	0	0	5	oxidized 11†	100
									reduced 5·3†	1
86.	Tetramethylthionine (methylene blue)	C	1	3	1	0	0	5	oxidized >11†	100
									reduced 5·8†	3

* Clark and Perkin (1932).
† Phillips, Clark and Cohen (1927).

A number of heterocyclic bases, belonging to nine series not previously discussed, have been sampled in Table V. It will be seen from Table V that none of these substances achieves high antibacterial activity. The reasons for this lack are dealt with further in the discussion.

DISCUSSION.

(i) *Heterocyclic Bases.*

This discussion is built around the two principles which the foregoing results indicate to be the most important limiting factors governing antibacterial activity in heterocyclic bases. These are (*a*) adequate ionization and (*b*) adequate area for the flat surface of the molecules.

It had been shown in Part II (Albert *et al.*, 1945) that a high degree of bacteriostasis in the acridine series occurs only in those members which are strong enough bases (as measured by the pK_a value) to ionize to the extent of at least 50 per cent at pH 7·3.

A similar correlation between bacteriostasis and ionization has now been demonstrated in a number of other heterocyclic series (Tables I, II and IV). The most obvious examples are those contained in Table I, which deals with the benzquinolines and phenanthridines (which are isomerides of acridine), and in Table II, which deals with benzacridines, which are higher benzologues of acridine (see illustrations at the head of tables). In the three isomeric series of benzacridines (Table II), the maximal antibacterial activities are equal to those commonly found among well-ionized acridines (e.g. 5-aminoacridine, No. 1 in Table I). In the other series (Table I) the highly ionized members often have much the same degree of activity as 5-aminoacridine towards Gram-positive species, but are relatively inactive towards the Gram-negative species tested here Actually a few acridines which behave in the same way were described in Part II, e.g., 2:5-diaminoacridine, 5-amino-1-phenylacridine and 2:8-*bis*dimethylaminoacridine. Three benzquinolines (Nos. 2, 7 and 17) were previously examined by Browning, Cohen, Gaunt and Gulbransen (1922) who found them inactive and concluded that the benzquinolines were in no way comparable with the acridines as antibacterials. It was unfortunate that their examples were all poorly ionized, although at the time neither the magnitude of the ionization constants nor the significance of ionization in bacteriostasis was known.

A similar mode of action may therefore be postulated for the acridines and the seven series described in Tables I and II. This similarity does not rest solely upon the basis of adequate ionization, because a practically complete lack of antibacterial power has been demonstrated in the *lower* benzologues of acridine (quinolines and pyridines, Table III). An apparent exception, 8-hydroxyquinoline, is antibacterial because of a peculiarity in its molecular architecture which enables it to chelate with metals (see Albert, Rubbo, Goldacre and Balfour, 1947). Some amino-quinolines and -pyridines (excluding all the highly ionized members) had previously been examined by Browning *et al.* (1922), with results similar to those reported here.

A likely explanation for this low activity is forthcoming from the results reported in Part II for 5-amino-6:7:8:9-tetrahydroacridine. It will be recalled that this substance was found to be almost lacking in antibacterial properties—a fact confirmed in the course of the present work (No. 61; Table IV). As pointed out in Part II, this substance, which at first sight differs from the highly antibacterial 5-amino-acridine (No. 1) only in the possession of four extra hydrogen atoms, owes its poor antibacterial properties to the loss of an important proportion of the flat surface of the molecule. Those molecules which, like 5-aminoacridine, are conjugated (i.e. every second bond is a double-bond) throughout are flat, virtually two-dimensional structures, hence all the atoms constituting the ring-system lie in the plane of the paper. However, any such molecule, when hydrogenated, loses its planar configuration. Such an effect can be seen in Fig. 1, where 5-aminoacridine and its tetrahydro-derivative are shown, built to scale with Herschfelder atomic models. It is seen from Fig. 1 that the molecule of 5-aminoacridine is planar enough to lie flat on a surface, but its tetrahydro-derivative cannot do so because of the distortion brought about by hydrogenation.

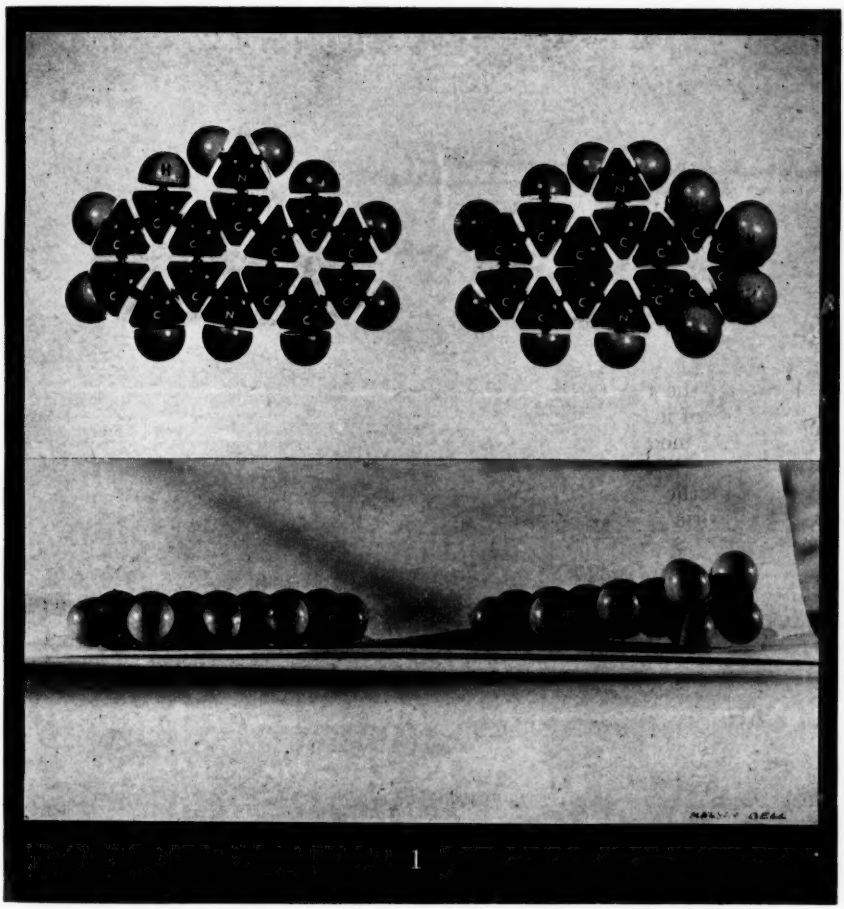


FIG. 1.—Loss of flatness on partial hydrogenation. Scale models of the ions of 5-aminoacridine (left-hand pair) and 5-aminotetrahydroacridine (right-hand pair).

5-Amino-6:7:8:9-tetrahydroacridine, having only two flat rings in the molecule, is actually nothing more than a simple aliphatic derivative of 4-aminoquinoline. As both are highly ionized, it would appear that any device which restored antibacterial action to the one would restore it to the other. Now it is seen from Table IV that the addition of another flat ring to 5-amino-6:7:8:9-tetrahydroacridine (to give 5-amino-6:7:8:9-tetrahydro-2:3-benzacridine, No. 62) has partly restored the activity. (The maximum activity possible here is that of 4-aminobenzquinoline (No. 15), which has a B.I. of 19). Similarly the addition of another flat ring to the various available positions of 4-aminoquinoline gives the highly active benzologues: 5-aminoacridine (No. 1), 4-amino-5:6-benzquinoline (No. 9), 4-amino-6:7-benzquinoline (No. 15) and 4-amino-7:8-benzquinoline (No. 21).

In order to confirm beyond all reasonable doubt the hypothesis of "insufficient area of flat surface", a method was sought of increasing the flat area of the quinoline molecule in some other way than by adding rings benzologously.

This cannot be done by adding a phenyl-substituent, giving for example 4-amino-2-phenylquinoline (No. 56). Although this substance has a sufficiently high degree of ionization, it is known that a phenyl group does not normally lie co-planar with a ring into which it is substituted, and that the expenditure of energy is required to force it into a co-planar state, as was first demonstrated for diphenyl by Karle and Brockway (1944), using electron-diffraction. It is seen from Table III that the provision of a phenyl-substituent has hardly increased the antibacterial power of 4-aminoquinoline any more than the provision of methyl-substituents (e.g. No. 55).

The problem of increasing the flat area of 4-aminoquinoline was solved by providing it with a (*trans*) styryl substituent giving, e.g., 4-amino-2-styrylquinoline (No. 64). It is well established in stereochemistry that the styryl-group takes up a position co-planar with the nucleus to which it is attached (*cf.* the work of Robertson (1935) and Robertson and Woodward (1937) on *trans*-stilbene, i.e. styryl-benzene). As may be seen from Table VI, the insertion of a styryl-group has enlarged the flat area of the quinoline molecule even beyond that of the acridine molecule. Naturally enough, this increase in area has no effect on the antibacterial properties when the operation is performed on a feebly ionized aminoquinoline, e.g. when 6-aminoquinoline is converted to 6-amino-2-styrylquinoline (No. 63). On the other hand, the effect of adding a styryl group to 4-aminoquinoline, which is well ionized, is to raise its antibacterial power from zero up to a figure comparable with the best of the benzquinolines, namely to a B.I. of 16. Further examples of this effect are seen in No. 65–68 (the δ-phenylbutadienyl-group in No. 68 is a higher vinylogue of the styryl group).

Similarly the addition of styryl-groups to 4-aminopyridine, giving 4-amino-2:6-distyrylpyridine (No. 69), has increased the antibacterial properties from zero to a high level. In assessing the activity of this substance it should be noted that the molecular weight is 298; hence it is 65 per cent heavier than any monoamino-benzquinoline or -acridine. As all the figures in this paper are reported on a weight- (as distinct from a molar-) basis, these heavier molecules (such as No. 69) are even more potent than the figures suggest.

These considerations of the necessity for a minimal flat area do not apply exclusively to heterocyclic molecules. It is evident from Table IV that strong antibacterial activity appears in two series (the aryl-guanidines and the aryl-diguanides) as soon as the flat area of the molecule corresponds to that of the

acridine and benzquinoline molecules (as in anthrylguanidine and anthryldiguanide, No. 72 and 75). It should be pointed out that although the guanidine and diguanide ions are themselves flat, spectrographic evidence has shown no conjugation between these groups and the aryl nuclei. Hence free-rotation, and not compulsory co-planarity, governs the relations of nucleus and side-chain in these cases.

Table VI has been compiled to assist quantitative appraisal of the flat-area effect. The dimensions of the nuclei have been calculated accurately from the tables of bond-distances given by Branch and Calvin (1941). The area of each nucleus has been considered approximately as the area of the minimal rectangular envelope that would fit around it, as shown in Fig. 2. The envelope has been located at a distance of 0·8 A exterior to the carbon skeletons (i.e. the contribution of the carbon atom to covalent bonds). Hydrogen atoms have not been included in these calculations, as they contribute only about one-tenth as much as the heavier atoms to a van der Waals' adsorption.

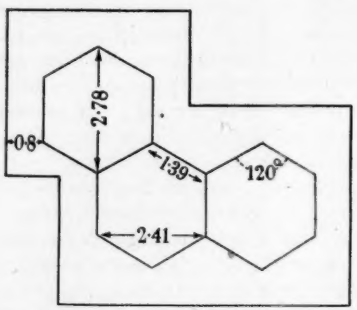


FIG. 2.—Minimal rectangular envelope fitting a phenanthridine or angular benzquinoline nucleus. Dimensions in Ångstrom units.

TABLE VI.—*Flat Areas of Molecules: Size of Minimal Rectangular Envelope which would contain Various Nuclei.*

Substance.	Dimensions in A.	Area in Sq. A.
Pyridine	(3·97 × 4·38)	17·4
Quinoline	(6·38 × 4·38)	27·9
Acridine also 6:7-benzquinoline	(8·78 × 4·38)	38·5
Phenanthridine, also 5:6- and 7:8-benzquinolines	..	38·3
2:3-Benzacridine	(11·16 × 4·38)	48·9
1:2- and 3:4-Benzacridines	..	48·7
Flat surface of tetrahydroacridine	..	(as quinoline)
Flat surface of tetrahydrobenzacridine	..	(as acridine)
2-Styrylquinoline	..	49·9
2:6-Distyrylpyridine	..	61·6
Phenyl-radical	..	(as pyridine)
Naphthyl-radical	..	(as quinoline)
Anthryl-radical	..	(as acridine)

It is seen from Table VI that the critical size, above which a flat kation becomes antibacterial, lies somewhere between the 28 sq. A of quinoline and the 38 sq. A of acridine. The necessity for this minimal flat area is obviously to provide a sufficiently great area for adsorption on to a biological surface.

Increasing the number of atoms in a molecule increases its chances of adsorption because the small van der Waals' forces, which unite any atom of the molecule to any atom of the bio-receptor surface, become collectively a really strong force when a large number of atoms are concerned on each side. This force is opposed by the kinetic energy of translation of the molecule, which does not increase as the size of the molecule increases. Hence there are critical sizes above which various molecules are likely to remain absorbed. The special requirement of the present series, viz. that the atoms responsible for this van der Waals' adsorption should all lie in one plane, strongly suggests that the atoms in the biological surface with which they have to make contact also lie in one plane.

The paramount requirement, that the molecule should be well ionized, proves that mere van der Waals' adsorption is not the biologically injurious act. As has been shown for the acridines in Part II, the biologically injurious act is a union (by salt-linkage) between the kationic portion of the drug (which for practical purposes may be considered as being, principally, the ring-nitrogen) and an anion which it is vitally important for the bacterium to have free. The function of the van der Waals' adsorption is to make more permanent the very transitory attachment effected by a salt-linkage in an ionic environment.

That there is no necessity for the kationic portion of the antibacterial substance to coincide with the area of van der Waals' adsorption is demonstrated by substances in which these two portions are at a distance from one another, e.g. anthrylguanidine (No. 72), anthryldiguanide (No. 75) and Ω-amino-5-ethylacridine (see Part II). However, activity of a really high order such as that shown by 5-aminoacridine and the highly-ionized benzacridines most likely depends not only upon the kationic portion falling within the area of van der Waals' adsorption, but even upon its occupying a particular part of that area.

Given these two essential requirements for antibacterial activity in heterocyclic bases, viz. adequate kationic ionization and an adequate area of flat surface, it is interesting to see what other heterocyclic families have these requirements. A survey has recently been made of the basic strengths of 23 other heterocyclic series (Albert *et al.*, 1948), and it has been found that almost all of the examples (which included the most basic amino-derivatives of each series) were such weak bases that one of the above requirements could not be fulfilled. The principal base-weakening feature in a heterocyclic molecule was found to be the presence of two hetero-atoms in the one ring. The almost complete lack of basic properties in nitrogen, when situated in a 5-membered ring, further reduces the choice of series. No. 76–84, in Table V, give a sample of this class of material. Most of the examples chosen also lack the minimal area of flat surface, being as poorly off as quinoline in this respect. As there is usually little variation in pK_a among benzologues, there is no reason to suppose that the addition of another ring to these substances (No. 78 possibly excepted) would make them active antibacterials.

Up to this point we have been discussing a high degree of ionization introduced by the phenomenon of additional ionic resonance (Part II), but two other methods are sometimes practicable, (*a*) a ring-nitrogen can be made quaternary, or (*b*) a basic side-chain can be built on to the molecule. Although the quaternization of a nitrogen atom can be relied upon to provide 100 per cent ionization in the aliphatic series, this is not a reliable device in heterocyclic series. Quaternary heterocyclic bases, when held at pH 7, usually undergo a chemical rearrangement

whereby an equilibrium is established between the original base and a derivative in which a hydroxyl-group has become covalently bound to a distant carbon atom, giving a "carbinol." As a consequence of this reaction an equilibrium pK_a is established, in some cases within a few seconds, in others after many hours. This new pK_a may prove too low to be useful, but even when this objection does not apply a fresh difficulty often arises, namely the carbinol autoxidizes to the corresponding (non-basic) ketone and hence the material is irretrievably wasted. Both of these difficulties were discussed in the case of methylacridinium salts in Part II. Yet, in spite of them, one moderately active substance was found which owed its high basic strength entirely to quaternization (3-amino-5:10-dimethylacridinium bromide), and a few other cases are known where antibacterial activity can be attributed to a successful introduction of a high degree of ionization by quaternization. Some moderately antibacterial quaternary styrylquinolines and quaternary phenazines have been described by Browning, Cohen, Ellingworth and Gulbransen, 1926, and Browning *et al.*, 1922, respectively. The non-quaternary analogues were inactive, which is not surprising, as they are now known to be poorly ionized.

The second alternative, insertion of a basic side-chain, can also cause difficulties, viz.: (i) An Ω-aminomethyl-group is often unstable on a highly conjugated ring; however, an Ω-aminoethyl- or a guanidyl-group is stable; (ii) Ω-aminoalkyl-groups are susceptible to destruction by amineoxidase; however, it is not established that this enzyme can operate on heterocyclic examples; (iii) biologically-inactive micelles may be formed by hydrogen-bonding from the ionized nitrogen of the side-chain to the non-ionized negatively-charged ring-nitrogen; this effect was encountered (and demonstrated potentiometrically) in 3-acridylguanidine and 3-acridyl-N^1-diguanide but it did not occur in 5-Ω-aminoethylacridine (it has also been encountered, in the absence of a basic side-chain, but for the same reasons, in 1-amino-*p*-phenanthroline).

One further general difficulty must be mentioned, although it may not often be encountered outside the thionine and oxonine series. These series have reduction potentials near to zero, so that they are easily reduced by a number of organisms. Thionine and methylene blue (No. 85 and 86) may be taken as examples. Although these molecules have the right basic strength and the necessary flat area, their E_0 values at pH 7 are + 0·06 and + 0·01 volts respectively. When reduced, their pK_a values are only 5·3 and 5·8 respectively (Phillips, Clark and Cohen, 1927), so that they then cease to be ionized. In the course of the bacteriological trials recorded in Table V, partial or complete reductions were recorded, which explains the low antibacterial values found. The acridine antibacterials have reduction potentials below — 0·47 volts (Part II), and those of the amino-benzquinolines, -acridines, -phenanthridines, -quinolines and -pyridines as well as various phenanthrolines and substances 76 to 83 in Table V fall well below this figure (J. B. Willis, private communication). Hence wastage of these substances through biological reduction is not to be expected.

In spite of the various difficulties outlined above, it should not be thought that the families listed in Tables I, II and IV exhaust the possibilities for finding new types of highly-active kationic antibacterials in the heterocyclic series. On the contrary, consideration of the above principles should help in the discovery of fresh examples.

TABLE VII.—*The Effect of Serum on the Antibacterial Action of Simple Aliphatic Amines. Highest Dilutions Completely Preventing Visible Growth in 48 hours at 37° C. (pH 7·2–7·4).*

Medium: A: Plain broth
B: 10 per cent serum broth

No.	Substance.	Medium.	Organisms. *Cl. welchii.*	*Str. pyogenes.*	*Staph. aureus.*	*B. coli.*	*Proteus.*	Bacteriostatic index (sum total of code numbers of inhibitory dilutions).	pK_a in water at 20° C.*	Per cent ionized (kation) at 20° C., pH 7·3.
87.	Undecylamine	A	4	5	3	3	2	17	10·7	100
		B	3	1	0	0	0	4		
88.	Dodecylamine	A	6	5	4	4	0	19	10·7	100
		B	1	0	0	0	0	1		
89.	Hexadecylamine (Cetylamine)	A	3	4	3	0	0	10	10·7	100
		B	3	3	3	0	0	9		
90.	Cetyltrimethylammonium bromide	A	5	6	6	1	0	18	>14	100
		B	4	5	3	0	0	12		

* Hoerr, McCorkle and Ralston (1943).

(iii) *Simple Aliphatic Amines.*

It is known that the antibacterial action of simple aliphatic amines increases as the pH of the medium is increased (Gershenfeld and Milanick, 1941). Acridines behave in the same way. It was shown in Part II that this is because the increasing alkalinity, although leaving the ionization of the acridine unchanged, increases the ionization of the weakly acidic groups—(from the data of Part II, the pK_a of these groups is above 9: They could be the — OH groups of tyrosine, purine or pyrimidine residues)—on the bacterial surface and hence facilitates the combination. It is pertinent to inquire whether the antibacterial action of these aliphatic bases, some typical examples of which are shown in Table VII, has anything further in common with the flat heterocyclic bases which we have been considering.

It has been shown that, as the chain length is increased, the aliphatic monoamines become highly antibacterial above the C_9 homologue (Fuller, 1942). Activity tends to fall off with higher members (about C_{16}), a fact which is apparently due to micelle-formation. Stereochemically, it should be noted that the aliphatic bases are not flat in any part; on the other hand, they are extremely flexible, whereas ring-systems possess a high degree of rigidity.

No attempt has yet been made to obtain a series of aliphatic bases of varying pK_a values and then to seek a connexion between ionization and antibacterial activity. The effect of increasing alkalinity makes it fairly certain that such a connexion would be found. However, an important difference between the aliphatic bases (Table VII) and the flat heterocyclic bases listed in Tables I to IV is that the former, when highly active antibacterially, are extremely susceptible to the presence of serum (*cf.* in particular No. 87 and 88, Table VII), but the latter are all unaffected by serum.

It is evident that the flat heterocyclic bases have a special feature which makes them less ready to combine with serum proteins but no less ready to combine with the vital receptors on the bacteria. This feature is, most likely, of a steric nature.

A key is attached for interconversion of the British system of numbering heterocyclic rings, as used in this paper, and the American system (Table VIII).

TABLE VIII.—*Key for Interconversion of the British System of Numbering (as Used in this Paper) and the American System.*

British Name.	American Name.		British numbering.												
			1	2	3	4	5	6	7	8	9	10	1′	2′	3′	4′
Acridine	Acridine		4	3	2	1	9	8	7	6	5	10	..	..	..	..
5:6-Benzquinoline	Benzo[f]quinoline	American numbering.	4	3	2	1	..	..	6	5	..	..	10	9	8	7
6:7-Benzquinoline	Benzo[g]quinoline		1	2	3	4	5	..	..	10	..	..	6	7	8	9
7:8-Benzquinoline	Benzo[h]quinoline		1	2	3	4	5	6	..	..	..	..	7	8	9	10
Phenanthridine	Phenanthridine		4	3	2	1	10	9	8	7	6	5	..	..	..	..
1:2-Benzacridine	Benz[c]acridine		..	..	5	6	7	8	9	10	11	12	1	2	3	4
2:3-Benzacridine	Benz[b]acridine		6	..	..	11	12	1	2	3	4	5	7	8	9	10
3:4-Benzacridine	Benz[a]acridine		6	5	..	..	12	11	10	9	8	7	4	3	2	1

SUMMARY.

1. Eighty heterocyclic bases having flat, rigid molecules have been examined for antibacterial activity (Tables I to V)

2. As with the acridines, antibacterial activity has been found to depend on a high degree of ionization at the pH of the tests (pH 7·3). It is shown that at least 50 per cent ionization at 20° C. (i.e. 33 per cent at 37° C.) is necessary before the full antibacterial potentialities of each series are approached (see particularly Tables I and II).

3. It has been shown that the molecules of heterocyclic bases, even when highly ionized, must possess a minimal flat area (above 28 sq. A) before powerful antibacterial properties appear (see Table IV). The acridines, the three series of benzquinolines, the phenanthridines and the three series of benzacridines all possess this requirement. Highly-ionized members of other series which fall below this requirement (quinolines, pyridines, aryl-guanidines and aryl-diguanides) are not normally antibacterial, but acquire activity when a sufficiently large co-planar area is built on to the molecule.

4. Other factors which may influence the antibacterial activity of heterocyclic bases are discussed and some comparisons with the simple aliphatic bases are made.

The authors wish to thank Dr. D. J. Brown and Dr. H. Duewell, and Mr. R. J. Goldacre, Mr. J. N. Phillips and Mr. R. P. Royer for their share in the chemical work which is embodied in various papers listed below, and without which this paper could not have been written. Our bacteriological colleagues, Miss M. E. Davey, Miss J. D. Stone and Miss D. M. Morris, are also thanked for their assistance in testing some of the substances. This work was partly supported by the National Health and Medical Research Council through a grant to one of us.—(M.I.B.)

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ANTIBIOTICS ACTIVE AGAINST BACTERIAL VIRUSES.*

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THE search for antibiotics against viruses requires new methods for detecting the presence of active substances in a reasonably short time, that is, before the product tested deteriorates. Direct tests with human and animal viruses are not yet suitable, being too slow and cumbersome for a routine screening method. On the other hand, bacterial viruses, i.e. bacteriophages, seem to be quite suitable for this purpose, provided that we make a totally unproved assumption that at least some of the animal and human viruses will be as sensitive to certain antibiotics as bacteriophage. There are no ways to prove or disprove such an assumption, even by analogy, until active antibiotics are actually found and tried. Furthermore, we are of the opinion that even if the antiphage substances discovered prove to be inactive against any of the other viruses, they will provide new interesting agents for the study of host-parasite relations as well as for the general study of cell biology. At the same time, from the technical point of view, bacteriophage is an ideal test virus giving test results in less than 24 hours, and it can be as easily handled as ordinary bacteria.

Therefore, our plan in search for antibiotics was to use bacteriophages as simulants of pathogenic viruses for screening purposes, for the follow-up of the production of such antibiotics, and for the basic studies of the mode of action of substances interfering with virus development.

* Aided by a grant from the Banting Research Foundation.

METHODS.

Mould cultures were usually isolated from plate contaminations and were not identified before trials.

Every mould tried was grown on four different media :

1. Papain meat digest (Papain broth) (Asheshov, 1941), pH 7·0.
2. Same with addition of 2 per cent sucrose.
3. A modification of Czapek-Dox medium :

Sodium nitrate	3 g.
Monopotassium phosphate	1 g.
Potassium chloride	0·5 g.
Magnesium sulphate	0·5 g.
Corn syrup	20 g.
Water	1000 ml.
Lactic acid, sufficient to bring the reaction to pH 4·5.	

4. Our own synthetic medium referred to as " No. 1 medium " :

Calcium lactate	1 g.
Monopotassium phosphate	1 g.
Ammonium nitrate	0·5 g.
Magnesium sulphate	0·25 g.
Corn syrup	20 g.
Water	1000 ml.
Lactic acid, sufficient to bring the reaction to pH 4·5.	

Media were distributed in 250 ml. amounts into Roux bottles and autoclaved at 118° C. for 20 minutes.

Flasks were inoculated by what we call " foam method," which differed depending on the nature of the medium inoculated. To papain broth a few ml. of a heavy suspension of mould spores were added. The bottles then were vigorously shaken until a thick layer of foam was produced. The foam, containing spores, disintegrated very slowly and disappeared only after the spores began to germinate. This fact ensured an even and dense film of surface growth. Synthetic media were inoculated differently. Since these media do not contain protein products, a solution of peptone deposited on the surface of such media immediately spreads over the whole surface in a thin film. Utilizing this fact, we prepared our spore suspension in either chilled broth or in peptone water. By means of a pipette, foam was produced of which one or two pipettes full (about 3 ml. in volume) were placed on the surface of the media, where it immediately spread over the whole surface. Such bottles, left undisturbed, yielded again an even and dense film of surface growth.

Samples of the culture media were collected as soon as the growth of the mould suggested that sufficient metabolites were present. Sampling was repeated at regular intervals, i.e. two or three times a week.

The samples were examined by the paper disc method on agar plates and, when necessary, by the serial dilution method in a special broth.

Paper disc method.

Plates for the paper disc method were prepared in the following way:

Medium:	Papain broth (Asheshov, 1941)	1000 ml.
	Bacto Yeast Extract	2·5 g.
	Agar ("K" by Agar Products Co., Los Angeles or Difco)	9 g.
	pH 7·0.	

The medium was sterilized only once and then distributed into petri dishes, 30 ml. per plate, using a Macalaster Bicknell 850 automatic pipette.

Just before use the plates were flooded with a suspension of bacteria, to which bacteriophage was added in sufficient numbers to produce almost confluent clearings. Special paper discs were soaked in the liquid to be tested and deposited on the surface of the plates. Two kinds of paper discs were used: large discs, $\frac{1}{2}$ in. in diameter, made by Schleicher & Schuell (740–E.), and small ones, $\frac{1}{4}$ in. in diameter, punched from filter-paper, S. & S. 598, with an ordinary office looseleaf punch. Small discs were used in testing new moulds, giving us an opportunity to examine 16 samples (four moulds on four different media) on one plate. The larger discs were used in testing samples already known to be active, thus providing a certain degree of quantitative estimation. Five to six discs could be used conveniently on one plate. After overnight incubation, provided the samples were active, the phage was suppressed and the discs were surrounded by an area of normal growth of bacteria, while the rest of the plate was covered by a layer of bacteria eaten out by the phage clearings (Fig. 1, 2 and 3).

Serial dilution method.

The following medium was used:

Papain broth	1000 ml.
Dextrose	10 g.
NaCl	8·5 g.
Brom Cresol Purple 0·1%	10 ml.
pH 7·0.	

It was distributed into 16 × 150 mm. tubes in 10 ml. for the first tube of the series and in 5 ml. portions for the rest of the series, and autoclaved.

Usually a two-fold dilution series of antibiotic was prepared. Then approximately one million bacteria per ml. and a number of phage corpuscles, sufficient to produce complete lysis in 18 hours, were added to each tube. This number was variable with different types and races of phage and with different strains of bacteria, and was determined experimentally before the test was run.

The results of the dilution test usually showed zonal effect. Since culture fluids and even purified fractions could have, beside antiphage action, also antibacterial effect, tubes containing higher concentrations could be devoid of bacterial growth, showing clear purple medium. With increased dilution the antibacterial effect would be eliminated. Those tubes would be turbid and yellow—bacteria developing freely as bacteriophage was suppressed. On further dilution, antiphage action, in its turn, would be eliminated—phage lysing the bacteria. These last tubes would be again clear and purple.

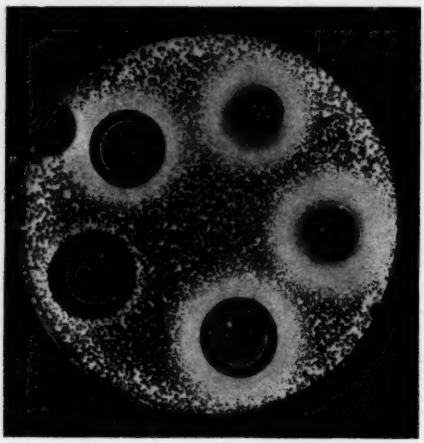

FIG. 1.

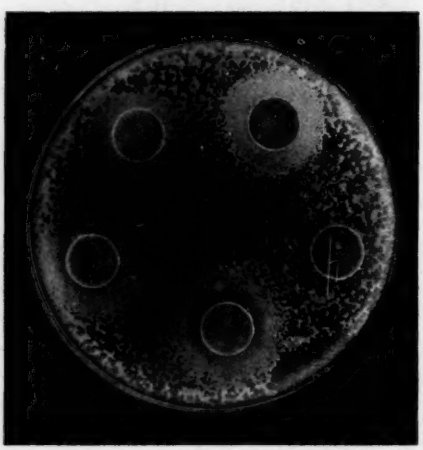

FIG. 2.

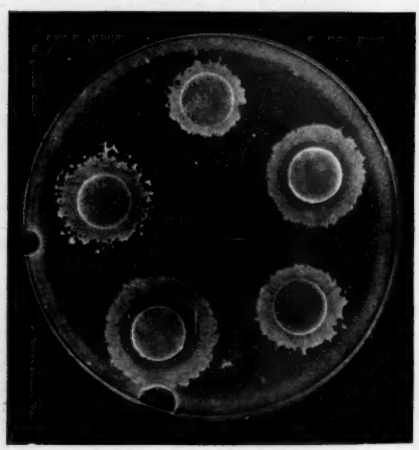

FIG. 3.

FIG. 1.—Assay on staphylophage. Two of the discs show combined action on staphylococci and on staphylophage. One disc shows almost pure antistaphylococci action. Two discs show pure antistaphylophage action.

FIG. 2.—Assay on streptophage. Clearings of the phage are almost confluent.

FIG. 3.—Assay on streptophage. Phage destroying all bacteria outside the influence of antibiotic in the discs.

The pH of the samples to be tested by disc or serial dilution methods was determined electrometrically. If the reaction fell below pH 6·0, it was brought up to about pH 7·0 by addition of a drop or two of a phosphate solution :

Potassium monophosphate .	10 g.
Phenol red 0·05% sol. . .	100 ml.

It was distributed into small test-tubes in 2 ml. portions and autoclaved. By adding phenol red, it was possible to see when approximate neutrality was reached.

At the beginning of the work we used as test viruses only one staphylophage and three coliphages, occasionally including phages of the *Bacillus* group (*B. cereus*, *B. megatherium*, *B. pumilis* and *B. brevis*). Later on, when the investigation showed considerable variations of sensitivity to antibiotics of different races and types of phages, we increased in our screening tests the number of different phages to 51. The following phages were used : 23 types of staphylophages ; 6 types of streptophages ; 7 types of enterococcus phages ; 14 coliphages ; 1 type of typhiphage and 1 *B. cereus* phage. Some of these phages were isolated by us. The majority were received from Dr. N. A. Boulgakov, Laboratoire du Bactériophage, Paris, France, to whom we express our sincere gratitude as well as our admiration for saving and maintaining such a valuable collection of bacteriophages through the entire war, German occupation, and the difficult post-war period. We are indebted to Dr. A. Schatz for three phage cultures, and to Dr. M. Delbrück for the T series of the *Bact. coli* phages.

By employing these methods we examined only 30 moulds in all. Early in the investigation we found a mould which produced very active antiphage substances and our search for new antibiotics became sporadic, time being consumed by the study of the newly-discovered substances. Only four other moulds showed some doubtful activity against some of the phages, but these have not yet been investigated any further.

The mould which showed considerable activity was an *Aspergillus* sp. It produced at least five different antibiotic substances at different stages of its development. Three of those substances were active only on bacteria, one active against some streptophages (to a lesser degree also against coliphages T2, T4 and T6), and one was active against several staphylophages.

THE PRODUCTION AND THE PROPERTIES OF ANTIPHAGE SUBSTANCES.

The antibiotic, active against several races of streptophages, is produced early in the development of the mould. Its activity can be demonstrated generally on the third or fourth day of growth. It reaches its peak between the 6th and 10th day, when the pH of the medium has dropped to its lowest level (approximately pH 3·5).

The active substance is extracted with chloroform from the culture fluid at alkaline reaction (pH approx. 10). The extract is concentrated, washed with dilute hydrochloric acid and treated with bentonite, which removes some inactive coloured substances. The chloroform extract is dried and the residue is dissolved in benzene. The benzene solution is extracted several times with dilute ammonia, and is subsequently treated with norit until it is colourless.

After the evaporation of the solvent, a gummy, pale yellow residue is left

which is almost insoluble in water, soluble in acetone, ether and most organic solvents except petrol ether.

It inhibits the development of streptophage in dilutions 1 in 10 to 1 in 20 million by weight. This represents a 1000- to 2000-fold purification of the starting material. The yield in most cases is close to 100 per cent.

By repeating the norit treatment of a more concentrated solution, using larger amounts of material, the activity can be increased to 1 in 40 million.

The substance is stable between pH 2 and 6. A slow decline in activity takes place at neutral and weakly alkaline reaction, while strong alkali (above pH 9) destroys it almost immediately.

Attempts to obtain the substance in crystalline form so far have been unsuccessful.

The second antibiotic, active against several strains of staphylophage, is produced during the third week of incubation. It is generally harvested between the 18th and 22nd day when the pH of the medium has returned to neutral. The culture fluid is acidified to pH 2 and the active substance is precipitated with phosphotungstic acid. The precipitate is dissolved in dilute ammonia, acidified and extracted with carbon tetrachloride. The active substance is extracted from this solvent with dilute ammonia. The aqueous solution is again acidified and extracted with benzene. The solvent is evaporated, the residue dissolved in dilute ammonia, acidified and extracted with ether. On evaporation of the solvent the active substance crystallizes in colourless, blunt-edged needles. They are washed with cold alcohol and recrystallized from hot 95 per cent alcohol. The crystalline material inhibits the development of staphylophages in dilution 1 in 20 million by weight, while approximately a hundred times higher concentration is required for a bacteriostatic effect on staphylococci. It does not show any action on streptophage or *Bact. coli* phage.

The substance is sparingly soluble in water, easily soluble in dilute alkali, and most organic solvents except petrol ether. It is fairly stable in aqueous solution between pH 4 and 9, but is slowly destroyed by strong acid and alkali. Heating to 100° C. for 15 minutes does not destroy its activity. It reduces permanganate in the cold and gives a yellow precipitate with $FeCl_3$. It is optically inactive. It has no sharp melting-point, but decomposes at approximately 160° C.

A more complete chemical characterization will have to await the accumulation of more material.

As soon as we were in possession of antibiotics against the two types of phages, i.e. an anti-staphylophage agent which was found first and an anti-streptophage agent found one year later, we began our investigation to determine the nature of the antiphage action. It was realized that the suppression of the bacterial virus could be the result of action on the virus itself, or on the host cell (bacterium), or on both. It could be possible that the antibiotic, without noticeably affecting the development of bacteria, affects their metabolism in such a way as to render them unsuitable as hosts for the virus, for instance, by blocking some enzyme system required by the virus. On the other hand, the antibiotic may influence the development process of the virus itself. It may also affect the liberation of the grown phage from bacteria by interfering with the lytic action of bacteriophage.

In an effort to elucidate these problems we began a series of experiments

presented in this paper, which do not yet solve the problem, but throw some light on the nature of the action of the antibiotic on the host-parasite system. The experiments were carried out with both antibiotics, and it is noteworthy that both showed a remarkable similarity in their action in spite of their entirely different chemical nature.

1. Is the bacteriophage corpuscle attacked by our antibiotics?—To a phage suspension, free from bacteria and containing about 1×10^8 phage particles, 50,000 units of antibiotic was added and was left to stand overnight at 37° C. If the highest active dilution of a liquid or of a dry fraction was, say, 1 : 500, we referred to it as containing 500 units. An identical suspension without antibiotic was used as a control. On the following day the phage particles present were recounted, any antibiotic action being eliminated by the dilution used. On no occasion was any decrease in the number of phage corpuscles noticed. We concluded that our antibiotics do not attack phage corpuscles, at least when they are in a resting or " dormant " state.

2. Does the antibiotic render the bacterial cell permanently unsuitable for phage development?—To determine this, bacteria were grown in the highest concentration of antiphage substance visibly not interfering with the normal development of bacteria. After 24 hours the bacteria were washed, resuspended in broth and inoculated with phage. The development of phage proceeded normally on the bacteria. This experiment indicates that the antibiotic does not induce hereditary damage to the bacterial cell.

3. Do our antibiotics interfere with adsorption of virus particles on bacteria? —Two identical suspensions of sensitive bacteria were prepared. Antibiotic was added to one of the suspensions in an amount not sufficient to influence the vitality of the bacteria, but exerting definite antiphage action—usually about 10 units. Both were then inoculated with phage. After several minutes, depending on the known adsorption time of the particular phage, both suspensions were centrifuged, the supernatants separated, and the sedimented bacteria resuspended in the same amount of broth. Supernatants and resuspended bacteria were plated for counting. Several different strains of staphylophage and streptophage, with their corresponding strains of bacteria, were treated in this way. On no occasion did we notice any interference with adsorption.

Examples :

Exp. 1. Staphylococci plus staphylophage plus 10 units of antibiotic. After 10 minutes 86 per cent of phage corpuscles were adsorbed on the bacteria in the presence of the antibiotic and 78 per cent in the control tube.

Exp. 2. Another strain of staphylophage with a different strain of staphylococci gave 73 per cent adsorption in the presence of the antibiotic and 80 per cent in the control tube.

Exp. 3. Enterococci phage. 10 units of antibiotic. After 10 minutes 100 per cent adsorption took place in the presence of the antibiotic and 95 per cent in the control tube.

Exp. 4. Coliphage T2, 2 units of antibiotic (the higher concentration had antibacterial action). After 10 minutes 100 per cent adsorption took place in the presence of the antibiotic and 97 per cent in the control tube.

It may be concluded from these experiments that our antibiotics, in concentrations several times stronger than those necessary to suppress the development

of phage, do not interfere with adsorption of the virus particles on bacteria, and therefore, in all probability, do not appreciably alter the surface of the bacterial cell.

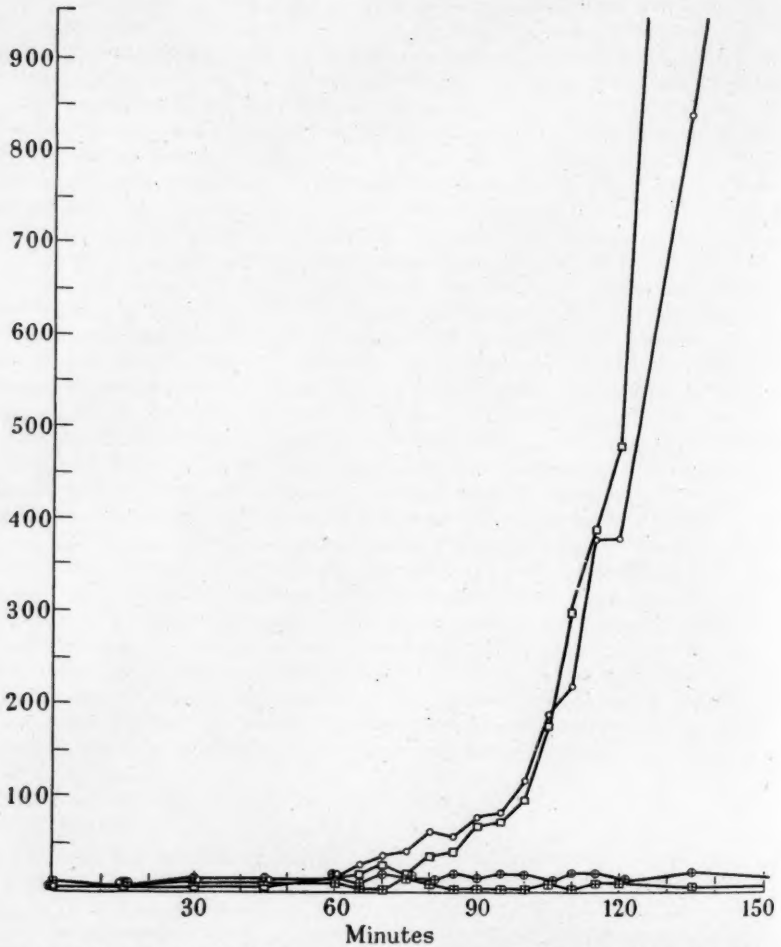

FIG. 4.—" Sensitive " staphylophage εφ18 developing on two different strains of staphylococci (ε4 and ε18) in presence and in absence of antiphage substance (αφ).

Tube 1. φ18/ε4 ..... □
Tube 2. φ18/ε4 + αφ ..... ⊠
Tube 3. φ18/ε18..... ○
Tube 4. φ18/ε18 + αφ ..... ⊕

4. Is the sensitivity of different races of phage influenced by the use of different strains of host cells?—We found an exceedingly variable sensitivity to our antibiotics among the 20 or more different staphylophages investigated.

Some phages were as sensitive as our test phage, some were almost completely resistant. As every staphylophage was propagated on its corresponding staphylococcus strain, we were able to decide, by following the development of a sensitive phage in the presence of antibiotic on the strain of staphylococci on which another phage proved to be resistant, whether the different sensitivity depended on the virus itself or on the host cell. By reversing the experiment we followed

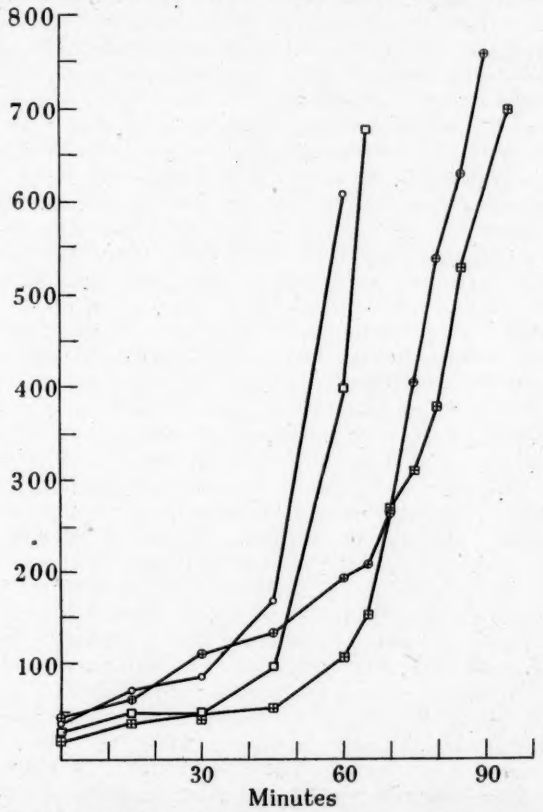

FIG. 5.—" Resistant " staphylophage εφ13 developing on the same two strains of staphylococci (ε4 and ε18) in Fig. 4 in presence and in absence of antiphage substance (αφ).

Tube 1. φ13/ε4..... □
Tube 2. φ13/ε4 + αφ ..... ⊠
Tube 3. φ13/ε18 ..... ○
Tube 4. φ13/ε18 + αφ ..... ⊕

the development in the presence of the antibiotic of a resistant phage on a bacterial strain on which another phage was suppressed. Several combinations were tried. Fig. 4 and 5 illustrate a typical experiment. In Fig. 4 a sensitive staphylophage Σφ18 shows normal development on two strains of staphylococci, Σ18 and Σ4, but there is no development on either of the strains of staphylococci

in the presence of 2·5 units of the antibiotic. When another staphylophage, Σφ13, was tried on the same two cultures (Fig. 5), its development was only slightly retarded by 2·5 units of the antibiotic in both cases. In other words, the resistance or sensitivity of a phage is maintained regardless of the strain of bacteria used to propagate it.

These experiments suggest, but do not prove definitely, that the action of the antibiotic is directed towards virus corpuscles and not towards the bacterial cell. The two phages, φΣ18 ("sensitive") and Σφ13 ("resistant") may require a different activity of the bacterial cell for their development, and our antibiotic may be suppressing only the one required by Σφ18, not affecting the cell process required by the "resistant" Σφ13.

5. Is the activity of the antibiotic against phage influenced by the number of bacteria used?—Several identical serial dilution sets of antibiotic were prepared, equal number of phage corpuscles were added to all sets, but a different amount of bacteria was used in each set. The end-point of the antiphage activity was the same in all sets.

6. Is the activity of the antibiotic influenced by the number of bacteriophage corpuscles used?—The previous experiment was repeated but an equal number of bacteria were used throughout the sets, the number of phage corpuscles being varied in each set. It was found that an increase in the number of corpuscles from 3×10^4 to 3×10^5 brought about a 50 per cent decrease in the activity of antibiotic, while a hundred-fold increase in the number of phage particles from 3×10^4 to 3×10^6 caused a 90 per cent decrease in the antibiotic activity.

7. Experiments were carried out to determine the influence of the addition of antiphage substance at different periods of phage development, but, before the first burst took place. A phage with a pre-bursting time of approximately 55 minutes was used. Five tubes with bacterial emulsion in broth were inoculated with bacteriophage. To one the antibiotic was added immediately after inoculation, the second received it after 15 minutes, the third after 30 minutes and the fourth after 45 minutes, i.e. 10 minutes before the bursts were expected to start. The fifth, or control tube, received no antibiotic. Only a few of the experiments carried out seemed to indicate that antiphage substance added at the time of infection, and 15 minutes after infection, prevented the bursts from taking place. Antibiotic added 30 minutes after infection probably allowed a few bursts to take place; there was a slight increase of the phage after one hour, but no further development occurred. Antibiotic added 45 minutes after infection did not prevent the first burst, but after that there was no further development. This experiment is of particular interest as it shows that, in all probability, our antibiotic interferes with the actual development of the phage either directly or indirectly and not with its liberation from bacteria.

8. Experiments also were conducted with the view to learn what would happen if a bacteria-virus complex submitted to the influence of antibiotic is later freed from the antiphage agent. All these experiments, performed with the crude metabolites (culture liquid) and not the purified substance, were not always consistent, probably due to the influence of other antibiotics present. However, the general tendency observed was that the phage, after having been freed from antibiotic, resumed its development, but only after a lag period. This lag period was never shorter than the pre-bursting time specified for each phage, but often longer. In some instances phage did not recover but, as

mentioned, this may have been due to the presence of impurities. These experiments again seem to support the point of view that the antiphage substance interferes with the actual process of growth, i.e. multiplication or formation of phage corpuscles inside bacteria.

Since the majority of the experiments described in this paper were carried out with either crude culture media or with only partly purified concentrates, they all are only of a preliminary nature and are to be repeated using purer substances when they will be available. At the same time it must be pointed out that the complexity of the phenomenon of the antiphage action is considerably greater than it seems at first glance. We have to deal with, observe, and study the living progress of two interdependent and continuously variable subjects—living bacteria and their living parasite, bacteriophage. We have learned only the elementary facts in regard to their relationships, in spite of some 30 years of study. Every step in our investigation requires additional study of the basic phenomena of the activity of bacterial viruses and, therefore, our progress is of necessity very slow. At the same time, our impression is that all these experiments are contributing to the better understanding of the biology of the host-parasite relationship in general.

SUMMARY.

Two different antibiotic substances active against some bacterial viruses were found to be produced by an *Aspergillus* sp. The procedure of their isolation and some observations on their action on bacteriophages are described.

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THE ERYTHROCYTE RECEPTOR FOR PERTUSSIS HAEMAGGLUTININ.

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IT has already been reported (Fisher, 1948) that saline suspensions of the acetone-soluble lipids of human erythrocyte stroma inhibit, in high dilutions, the action of pertussis haemagglutinin, and that the same suspensions act as lipid haemagglutinins, i.e. they agglutinate the erythrocytes of certain fowls (Burnet and Stone, 1946). This paper deals with further work aimed at identification of the active principles, which will be referred to as "inhibitor" and "agglutinin."

MATERIAL AND METHODS.

Acetone soluble erythrocyte lipids.

Mixed, unwashed, lyophilized human erythrocytes were extracted with boiling acetone, using either a continuous process, or three successive lots of solvent.

The acetone soluble lipids were recovered by evaporation of the solvent on the water bath.

Pertussis haemagglutinin.

Virulent pertussis organisms were extracted with M/1 NaCl (Keogh, 1948, unpublished), the organisms removed by centrifuging, and the clear supernates adjusted to contain 8 minimal haemagglutinating doses per drop. In some experiments the supernates of centrifuged fluid cultures were used as the source of haemagglutinin.

Inhibitor and agglutinin.

Lipoid suspensions were prepared, and inhibitor and agglutinin titrations set up, as described in the previous paper (Fisher, 1948). Agglutinin titres are expressed as the reciprocal of the dilution, and inhibitor titres as the reciprocal of the dilution multiplied by the number of minimal doses of haemagglutinin used.

RESULTS.

The acetone extracts of erythrocytes yielded on evaporation a brown oily fluid from which a large amount of white solids separated on standing. Nitrogen could not be detected by the sodium fusion test. The phosphorus content was 0·5 per cent, corresponding to 12·5 per cent of phospholipid. (Phospholipids are somewhat soluble in hot acetone; Bloor, 1943.) The acetone-soluble material could be separated by extraction with aqueous methanol into fractions which were active and inactive in respect of inhibition and agglutination. The portion soluble in 85 per cent and that insoluble in 95 per cent methanol were both inactive, whereas the material insoluble in 85 per cent but soluble in 95 per cent methanol was highly active. The two inactive fractions, when re-combined, exhibited the high activity of the original acetone extract.

In one experiment 400 mg. of acetone soluble material was extracted with two 15 ml. lots of 85 per cent aqueous methanol. The insoluble material was removed by centrifugation, and on evaporation of the solvent on the water bath, 176 mg. of amorphous greenish-brown material was recovered (Fraction 1). The insoluble residue was extracted with two 15 ml. lots of 95 per cent aqueous methanol, and by evaporation of the solvent, after removal of insoluble material by centrifugation, 54 mg. of yellowish mixed amorphous and crystalline material was obtained (Fraction 2). The residue of almost white, mostly crystalline material weighed 175 mg. (Fraction 3).

The results of quantitative Liebermann-Burchard tests on each fraction expressed as g. cholesterol/100 g. were: Fraction 1, 12 per cent; Fraction 2, 53 per cent; and Fraction 3, 78 per cent.

Approximate estimations of unsaponifiable material were done on Fractions 1 and 3 by the following method. Samples (20–30 mg.) were boiled in 15 ml. of aqueous 1 per cent KOH for 5 minutes in a nitrogen atmosphere. The samples were cooled and extracted several times with diethyl ether and petroleum ether. The extracts were combined, the solvents evaporated, and the residue weighed. Fraction 1 contained 10 per cent and Fraction 3 90 per cent of unsaponifiable material.

Saline suspensions of each fraction and of combinations of them were prepared

from their solutions in diethyl ether and tested for inhibitory and agglutinating activity. The results are shown in Table I.

TABLE I.—*Aqueous Methanol Fractionation of Acetone-Soluble Lipids of Erythrocytes.*

Fraction.	Solubility in aqueous methanol.	Inhibitor titre.	Agglutinin titre.
1	Soluble in 85%	$<10^4$	$<10^4$
2	Soluble in 95%; insoluble in 85%	$1{\cdot}6 \times 10^6$	$1{\cdot}6 \times 10^5$
3	Insoluble in 85% and 95%	10^5	$<10^4$
Mixture of equal parts of Fractions 1 and 3		$3{\cdot}2 \times 10^6$	$1{\cdot}3 \times 10^6$

The bulk of the saponifiable material, most of the pigment and all the phosphorus were contained in Fraction 1, soluble in 85 per cent methanol. This fraction suspended well in saline, but was inactive as agglutinin or inhibitor.

Fraction 3, insoluble in 95 per cent methanol, consisted mainly of unsaponifiable material, chiefly steroid. It gave poor saline suspensions, which were only weakly active.

A mixture of these inactive fractions 1 and 3 gave good saline suspensions of high activity.

Fraction 2, which physically and chemically was intermediate in composition between Fractions 1 and 3, gave well-dispersed saline suspensions of high activity.

These results indicated that the inhibitory and agglutinating activities of the acetone-soluble portion of erythrocyte stroma were properties of a sterol, only slightly soluble in aqueous methanol, which required to be associated with saponifiable lipoids, relatively soluble in aqueous methanol, for exhibition of its full activity.

In view of these findings, combinations of cholesterol with lecithin, and with oleic acid, were examined. Saline suspensions were prepared from diethyl ether solutions and tested for inhibitory and agglutinating activity. The results are shown in Table II.

TABLE II.—*Agglutinin and Inhibitor Titres of Suspensions of Cholesterol Containing Lecithin or Oleic Acid in Varying Proportions.*

(a) *The lecithin-cholesterol series.*

Reciprocal of (concentration $\times 10^4$)		Quality of dispersion.	Agglutinin titre.	Inhibitor titre.
Cholesterol.	Lecithin.			
—	1	Good	—	—
16	1	,,	$<1{\cdot}6 \times 10^5$	$1{\cdot}0 \times 10^6$
8	1	,,	$<0{\cdot}8 \times$,,	$2{\cdot}0 \times$,,
4	1	,,	$<0{\cdot}4 \times$,,	$6{\cdot}4 \times$,,
2	1	,,	$12{\cdot}8 \times$,,	$12{\cdot}8 \times$,,
1	1	,,	$12{\cdot}8 \times$,,	$12{\cdot}8 \times$,,
1	2	,,	$6{\cdot}4 \times$,,	$6{\cdot}4 \times$,,
1	4	,,	$3{\cdot}2 \times$,,	$3{\cdot}0 \times$,,
1	8	Fair	$2{\cdot}4 \times$,,	$1{\cdot}0 \times$,,
1	16	,,	$1{\cdot}0 \times$,,	$0{\cdot}5 \times$,,
1	—	Poor	$0{\cdot}05 \times$,,	$0{\cdot}25 \times$,,

TABLE II—*continued.*

(b) *The oleic acid-cholesterol series.*

Reciprocal of (concentration × 10^4). Cholesterol.	Oleic acid.	Quality of dispersion.	Agglutinin titre.	Inhibitor titre.
—	1	Good	(1 × 10^5)*	(0·1 × 10^6)*
128	1	,,	150 × ,,	6·0 × ,,
64	1	,,	70 × ,,	6·0 × ,,
32	1	,,	60 × ,,	6·0 × ,,
16	1	,,	20 × ,,	6·0 × ,,
8	1	,,	20 × ,,	12·0 × ,,
4	1	,,	20 × ,,	7·7 × ,,
2	1	,,	10 × ,,	4·8 × ,,
1	1	,,	5 × ,,	3·6 × ,,
1	2	,,	6 × ,,	3·0 × ,,
1	4	Fair	20 × ,,	1·8 × ,,
1	8	,,	5 × ,,	0·9 × ,,
1	—	Poor	0·08 × ,,	0·05 × ,,

* Refers to titre in terms of oleic acid concentration. Titres are based on cholesterol concentrations, except in the cases noted.

In the lecithin-cholesterol series the agglutinin and inhibitory activities showed a maximum in the same region, where the components were in equal concentration, or where lecithin was in slight excess. In the oleic acid-cholesterol series conditions were more complex, since oleic acid itself has haemolytic and haemagglutinating properties. In both series the inhibitory activity was slight in the region of excess cholesterol.

Palmitic acid and cephalin tested under the same conditions as oleic acid and lecithin failed to enhance the slight inhibitory action of saline suspensions of cholesterol.

Several sterols and steroid derivatives were tested to determine which part of the sterol molecule is active in inhibition. The substances were dispersed in saline from their solutions in diethyl ether or ethanol, in combination with oleic acid or lecithin. Cholic and desoxycholic acids were dissolved in saline with the aid of a little alkali. The results are shown in Table III.

TABLE III.—*Inhibitory Power of Some Steroids against Pertussis Haemagglutinin.*

Compound.	Inhibitory titre.
Cholesterol	10^7
Ergosterol	3×10^6
Calciferol	3×10^6
Corn germ sterol	6×10^6
Androsterone	$<10^5$
Cholesterol palmitate	$<10^5$
Cholesterol oleate	$<10^5$
Cholic acid	$\vee 10^5$
Desoxycholic acid	10^5
7-keto-cholesterol acetate	10^5

The substances which were active inhibitors, cholesterol, ergosterol, calciferol and corn germ sterol (presumably mainly stigmasterol), differ from the inactive substances in having three structural properties in common—(1) free hydroxy-group at C3, (2) double bond between C5 and C6, (3) presence of a side-chain.

The same substances, except cholesterol oleate, were tested for their ability to inhibit the haemolytic action of digitonin, by a technique similar to that of Ponder (1945). Those substances which inhibited pertussis haemagglutinin also inhibited haemolysis by digitonin, and those inactive against digitonin failed to prevent haemolysis.

DISCUSSION.

The above results indicate that cholesterol is the inhibitor of pertussis haemagglutinin present in acetone extracts of erythrocytes. The total amount of inhibitor which can be extracted from erythrocytes corresponds approximately to their cholesterol content, and cholesterol in association with lecithin or oleic acid inhibits to the same degree as the acetone soluble lipids. The evidence that the inhibitor present in acetone extracts of erythrocytes is also the receptor on the cell surface was discussed in the previous paper (Fisher, 1948). The work of Stone (1946) shows that the lipid haemagglutinin is of similar nature, and our results are in conformity with her findings.

Previous failure to recognize cholesterol as the inhibitor (Fisher, 1948) was due to two circumstances. First, the accessory but essential role of associated lipids in the preparation of cholesterol suspensions of maximum inhibitory titres had not been elucidated. Secondly, it was not appreciated that digitonin may fail to precipitate cholesterol quantitatively from solutions of the acetone soluble lipids of erythrocytes. Using the method of Kelsey (1939), it has since been observed that the percentage removal of inhihibitor from such material may vary from *nil* to 90 per cent.

Ponder (1945) has shown that the active patch for digitonin fixation in the steroid molecule is the C3-C5-C6 region. The evidence presented suggests that the same region is concerned in fixation of pertussis haemagglutinin, but the possibility has not been excluded that the nature of the side-chain may have a modifying action.

SUMMARY.

1. The inhibition of the haemagglutinin of *H. pertussis* by the acetone-soluble lipids of human erythrocytes appears to be due to the cholesterol contained in the lipids.

2. Cholesterol in saline suspension does not exhibit its full potential inhibitory action against pertussis haemagglutinin in the absence of the saponifiable portion of the acetone-soluble lipoids, for which lecithin or oleic acid may be substituted.

I am indebted to Dr. P. Fantl for the gift of several samples of sterols and sterol derivatives.

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ENZYME ADAPTATION IN BACTERIA: FATE OF NITRATASE IN NITRATE-ADAPTED CELLS GROWN IN THE ABSENCE OF SUBSTRATE.

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SOME progress has been made in the study of enzymatic adaptation in micro-organisms by following the increase in specific enzyme activity which may occur on incubation or growth of cells in the presence of substrate. The reverse process of "deadaptation," however, has not yet been properly investigated. Although it is known that in many cases a single subculture of previously-adapted cells in a medium not containing the substrate is sufficient to cause loss of adaptation, little systematic work has been reported upon the nature or progress of this reversion.

The autocatalytic nature of the curve of most enzymatic adaptations in washed suspensions of cells has led Spiegelman (1946) to suggest that an adaptive enzyme or its precursor might act under some circumstances as a self-reproducing entity. On the other hand, Spiegelman and Dunn (1947) have brought forward evidence supporting the concept of enzyme "interaction"—viz. formation of a new adaptive enzyme at the expense of previously-formed enzyme when the substrate responsible for the first adaptation is replaced by a second substrate.

The behaviour of an adaptive enzyme in adapted cells growing and multiplying in a medium without the substrate known to be necessary for maximal enzyme production must presumably correspond to one of the following three hypotheses:

(1) The enzyme activity per cell may remain constant, or fall off slowly, such that there is an increase in the total amount of enzyme in the culture.

(2) The preformed enzyme may be destroyed or inactivated so that the total enzyme in the culture diminishes.

(3) The total enzyme may remain constant so that, since the cells are growing, the enzyme activity *per cell* will gradually decrease, and the deadaptation process will eventually be achieved by a simple "dilution out" of the adapted enzyme.

If (1) were true, it would suggest that the enzyme or enzyme precursor, induced by substrate might be endowed with some independent self-reproducing properties, and would in any case be evidence in support of the direct effect of environment upon heredity. Hypothesis (2) is what might be expected in cases where adaptive enzyme interaction (Spiegelman and Dunn, 1947) occurred. Hypothesis (3) is what would occur if preformed enzyme remained

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unmodified—being neither inactivated nor "growing" with the cells—during increase of the rest of the bacterial protoplasm.

The adaptation of the nitratase enzyme system has been studied in the presence of nitrate in non-proliferating washed suspensions (Pollock, 1946) and during growth (Pollock and Wainwright, 1948) using the Intermediate coliform bacterium "1433." The same cells can be adapted simultaneously and independently to tetrathionate reduction (tetrathionase); and tetrathionate acts equally well as nitrate as H-acceptor for anaërobic growth with lactate or glucose. It was, therefore, decided to study the problem of deadaptation by following the nitratase of nitrate-adapted cells inoculated into a medium containing tetrathionate: that is, during growth in, and further adaptation to, a new substrate. The problem was slightly complicated by the fact that there is always a certain small amount of nitratase activity developed, even though the cells are grown without substrate. This, in practice, means that the total nitratase of a growing culture is always increasing, even in the absence of nitrate. However, allowance can be made for this "basal" nitratase, and the results here reported show that the behaviour of adaptively formed nitratase corresponds closely with that which would be expected were the loss of adaptation to proceed by a process of "dilution out" of the preformed enzyme (Hypothesis 3).

METHODS.

Organism.—"1433"—a lactose-fermenting "Intermediate, Type I" strain of *Bact. coli* (Pollock, 1946).

Suspensions.—Prepared as previously described (Pollock and Wainwright, 1948) from the 16-hour growth at 35° on tryptic meat agar.

Adapted suspensions were prepared by incubation for 3–3½ hours at 35° in M/100 glucose, M/50 sodium nitrate (or tetrathionate), M/5 phosphate buffer, pH 7·2 and M/600 $MgSO_4$, at a cell strength of 1 mg./ml. The cells were then twice washed with distilled water. Control unadapted suspensions had similar treatment, but without nitrate or tetrathionate.

Nitratase activity was followed at 35° in evacuated Thünberg tubes with M/100 sodium nitrate and M/50 formate, as H-donor, in the presence of M/800 $FeSO_4$. Samples were removed at 30 min. and 60 min. and the nitrite was estimated by means of the Griess Ilosvay Reagent (Pollock, 1946). Results are expressed as μmol. nitrite formed/mg. dry weight of cells/hour. Cell strength was determined by opacity measurements on the washed cell samples.

Tetrathionase activity was estimated by iodine titration of thiosulphate formed during incubation of cells anaërobically, at a concentration of 1 mg./ml. in Thünberg tubes, at 35° with M/100 sodium tetrathionate and M/50 formate, as H-donor (Pollock and Knox, 1943). Results are expressed as μmol. thiosulphate formed/mg. dry weight of cells/hour. *"Total" nitratase or tetrathionase activities* simply refer to the total amount (in μmol.) of nitrite or thiosulphate formed by the whole bacterial culture per hour.

Growth medium for deadaptation experiments was as follows:

M/5 phosphate buffer, pH 7·2, M/50 glucose, M/50 H-acceptor (sodium tetrathionate or sodium fumarate), M/100 NH_4Cl and M/600 $MgSO_4$. Growth was followed anaërobically at 35° in 6 × ¾ in. tubes, filled with argon (as described by Pollock and Wainwright, 1948). Up to 8 similarly prepared and inoculated

tubes were used for each curve, one tube being used for each sample. Initial concentration of cells was 50 μg./ml.

Samples for nitratase determinations.—1 ml. of M/200 oxine (8-hydroxyquinoline) was added to a whole tube of 10 ml. to stop growth and further adaptation. The cells were centrifuged, washed once with water, made up to about 1·0 ml. and the concentration estimated turbidimetrically.

Growth was followed in the tubes by direct estimation of opacity on the "Spekker" absorptiometer by means of a specially-constructed tube adaptor (Pollock and Wainwright, 1948). A standard curve of the relation between dry weight and opacity was prepared, and all results are given in terms of dry bacterial weight.

RESULTS.

Absence of enzyme "interaction" in washed suspensions.

As mentioned above, previous experiments (Pollock, 1946) had suggested that it was possible to further adapt nitrate-adapted cells of "1433" to tetrathionate without significant loss of the original nitratase, and *vice versa*. In this respect, behaviour is quite different from washed suspensions of *Saccharomyces cerevisiae* adapted to galactose which lose galactozymase activity on being adapted, under similar conditions, to maltose (Spiegelman and Dunn, 1947). Table I gives the results of a more extensive confirmatory experiment, and shows

TABLE I.—*Independence of Adaptations to Nitrate and Tetrathionate by Washed Suspensions of* "1433."

Cells previously adapted to:	Cells further treated anaërobically for 3 hr. at 35° at a conc. of 1 mg./ml. with: M/5 phosphate buffer (pH 7·2) M/600 $MgSO_4$ M/10 glucose.	M/10 sod. nitrate.	M/10 sod. tetrathionate.	Nitratase activity.	Tetrathionase activity.
Tetrathionate	+	+	—	3·9	8·4
	+	—	—	0·2	7·4
	No further treatment		..	0·2	8·6
Nitrate	+	—	+	3·8	6·5
	+	—	—	3·8	0·3
	No further treatment		..	4·1	0·2

that there is no evidence of "interaction" between tetrathionase and nitratase in washed suspensions. The final enzyme activities attained with respect to both nitratase and tetrathionase differ little whichever substrate was added first. The further treatment of nitrate-adapted cells to tetrathionate does not significantly diminish the original nitratase activity, and *vice versa*. Moreover, previously adapted cells appear to adapt as well to their second substrate as do unadapted cells to their first. Both enzyme adaptations appear to proceed independently.

The next stage was thus clearly to determine if such independence persisted during growth and cell multiplication. Here, however, it was only possible to follow the nitratase activities because the iodometric determination of thiosulphate is not sensitive enough to enable the tetrathionase activities of low concentrations of cells to be estimated accurately.

Experiments with growing cells.

Most of the experiments were done with tetrathionate as H-acceptor for reasons given above; but, in order to determine whether cells behave similarly in other media, in one experiment the tetrathionate was replaced by M/50 fumarate.

Two difficulties were encountered from the beginning:

(1) The problem of measuring nitratase activities of samples of cells at a particular point during growth.

(2) A "dilution effect." Both dilution of cell suspensions below a concentration of 0·2 mg./ml. and the centrifugation of cells from large volumes of fluid during washing caused considerable loss of nitratase activity. This inactivation was variable in extent and not nearly so marked, in unadapted cells. A similar sort of "dilution phenomenon" has been reported in the case of other enzyme systems and was found for the nitratase *adaptation* process (Pollock and Wainwright, 1948), although in the latter case it first became apparent at a higher concentration of cells than for the nitratase enzyme system itself.

The first problem was fairly easily overcome by the addition of M/2200 oxine to the growing culture. Fig. 1 illustrates an experiment in which two lots of cells were grown in 10 ml. of tetrathionate medium in Thunberg tubes containing 1 ml. of water and 1 ml. of M/200 oxine, respectively, in the stoppers. After 4 hours' growth the contents of the stopper were tipped into the main tube. Growth continued in the culture to which water had been added (Curve B), while it was almost completely and immediately arrested by the oxine (Curve A) for at least an hour. Nitratase adaptation was also completely inhibited by this concentration of oxine, but the enzyme itself was quite unaffected. After addition of the oxine, the cells were washed once with water before being tested for nitratase activity. It was confirmed that the nitratase activity of cells treated in this way with oxine did not differ significantly from that of cells similarly treated but without oxine; and it seems reasonable to assume that the nitratase activity of samples from a growing culture measured in this way must correspond very closely with the true enzyme activity of the cells in the growth medium at the time of adding oxine.

The second difficulty was largely, though not completely, overcome by the addition of M/800 freshly prepared $FeSO_4$ to the suspension mixture during measurement of nitratase activity. Table II shows that the loss of activity on dilution and/or washing was very considerably reduced by Fe^{++}, but that some stimulation of nitratase in unwashed, undiluted cells (Expt. 1 and 2)

TABLE II.—*Inactivation of the Nitratase of Nitrate-Adapted Cells caused by Washing at Low Concentration, and its Reversal by Fe^{++}.*

	Unwashed Cells.			Cells twice washed at a strength of 50 μg./ml.		
		Nitratase activities.			Nitratase activities.	
Expt. No.	Final conc. cells μg./ml.	Without Fe++.	With Fe++.	Final conc. cells μg./ml.	Without Fe++.	With Fe++.
1.	200	3·8	4·4	50	1·9	3·1
2.	200	4·4	4·8	28	1·1	5·3
3.	48	1·6	3·7	36	0·3	2·2

occurred with Fe^{++}, and the extent of reactivation was variable, being complete in Expt. 2, but only 60 per cent in Expt. 3. Repeated attempts to obtain more consistent figures were not successful, and it must be admitted that results given for nitratase activity shortly after inoculation, where cell concentrations used in the enzyme assay were low (usually about 100 μg./ml.), may be subject to some error. Nevertheless, it is not felt that such possible inaccuracies seriously affect the general picture given by curves relating growth and enzyme activity, and these experiments have been repeated many times with very similar results.

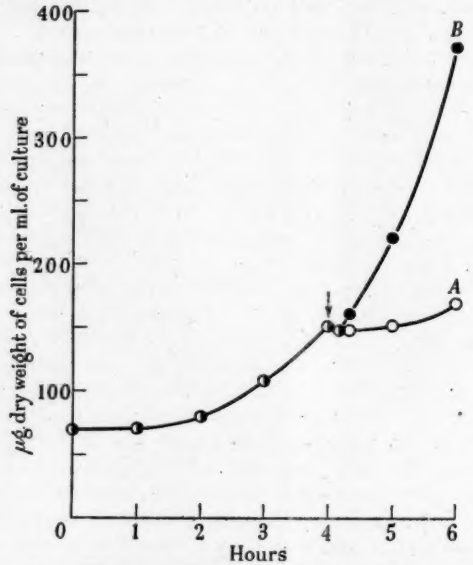

FIG. 1.—Effect of M/2200 oxine on growth of "1433" in tetrathionate medium. Additions were made to the culture at the point indicated by the arrow (see text). A, Oxine added. B, Water added.

A slight precipitate formed on addition of the Fe^{++}, but the estimation of nitrite was not interfered with in any way. It appears likely that this "dilution" phenomenon is due to a more or less completely reversible oxidation of some component in the system; similar but less satisfactory results could be obtained with other reducing agents such as cysteine, and glutathione.

Results have been summarized in Fig. 2 and 3, showing the relation between nitratase activity per mg. dry weight of cells, total nitratase of the culture and growth, expressed as dry weight, of adapted and unadapted cells growing in the different media. For comparison, results obtained in a nitrate-containing medium are shown in Fig. 4. The period covered by these studies allows for a maximum of only a 10-fold increase in cell mass—probably about three cell generations; but even this relatively small amount of growth is sufficient for the nitratase activity of previously adapted cells to have approached that of the unadapted

cells. There is no significant difference between growth rates of adapted and unadapted cells in media without nitrate; although, of course, in the nitrate medium, nitrate-adapted cells start growing appreciably sooner, as shown previously (Pollock and Wainwright, 1948).

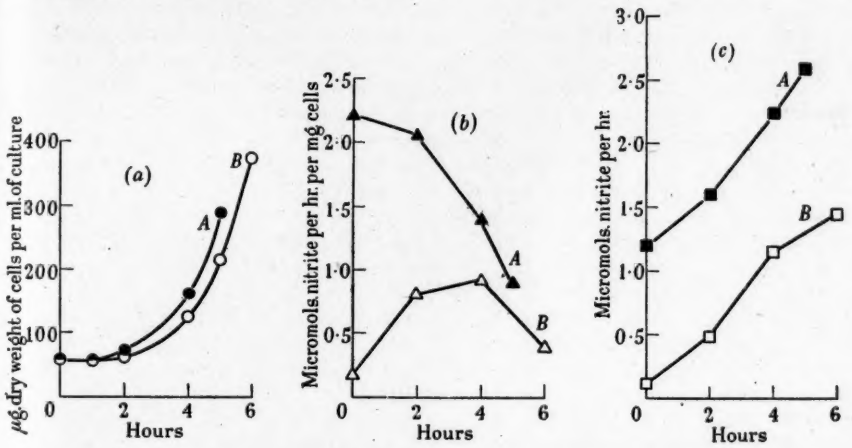

FIG. 2.—Variation of nitratase activity of nitrate-adapted and unadapted cells during growth in tetrathionate medium. A, Adapted cells. B, Unadapted cells.
(*a*) Growth curves.
(*b*) Nitratase activity of cells.
(*c*) Total nitratase activity of culture.

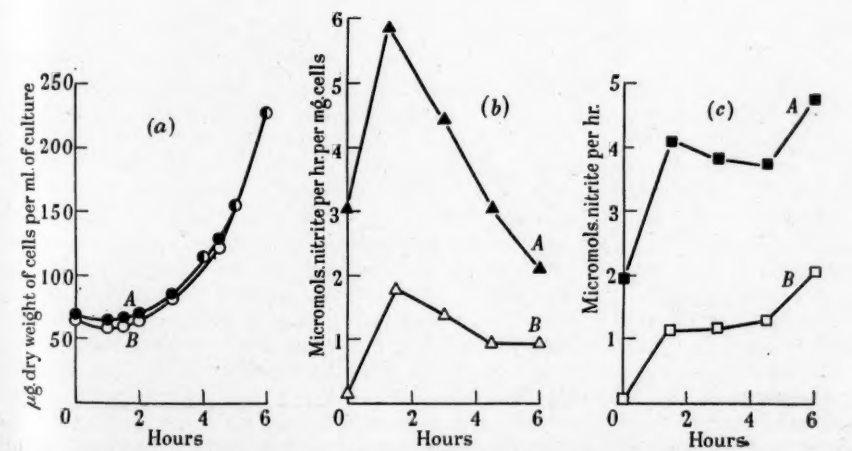

FIG. 3.—Variation of nitratase activity of nitrate-adapted and unadapted cells during growth in fumarate medium. A, Adapted cells. B, Unadapted cells.
(*a*) Growth curves.
(*b*) Nitratase activity of cells.
(*c*) Total nitratase activity of culture.

It will be noted that there is quite a considerable, though variable, amount of "basal" nitratase formed by unadapted cells growing without nitrate, but that the excess total nitratase of the adapted cells over that of the unadapted cells remains very roughly the same at all stages of growth, and corresponds to

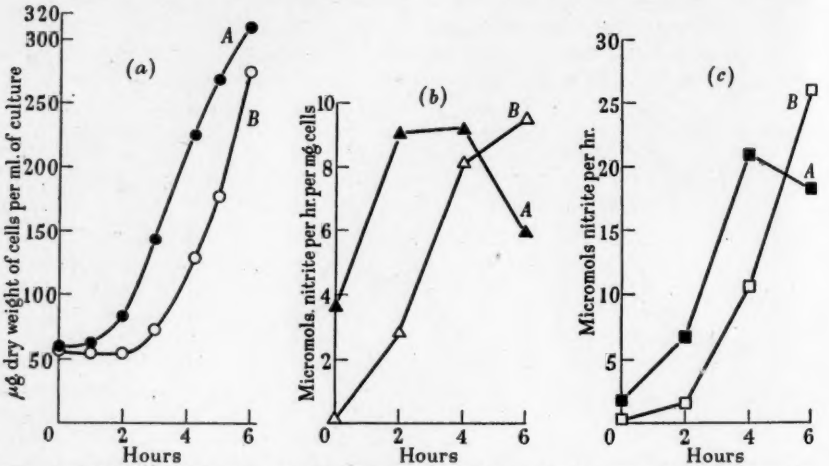

FIG. 4.—Variation of nitratase activity of nitrate-adapted and unadapted cells during growth in nitrate medium. A, Adapted cells. B, Unadapted cells.
(*a*) Growth curves.
(*b*) Nitratase activity of cells.
(*c*) Total nitratase activity of culture.

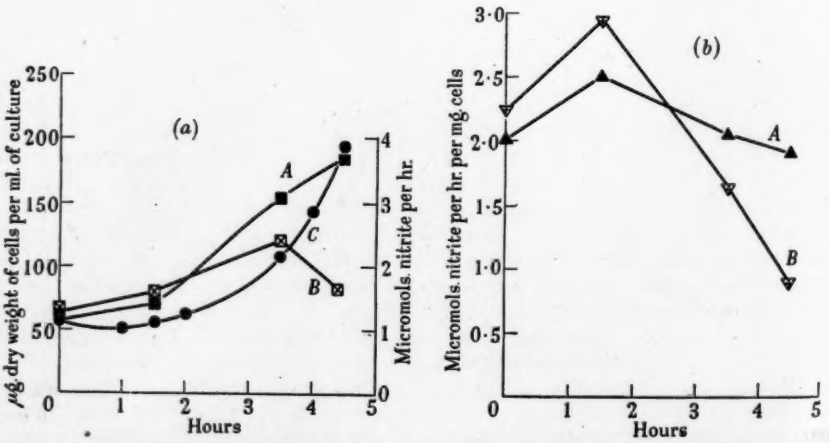

FIG. 5.—Variation of nitratase activity of nitrate-adapted cells during growth in tetrathionate medium as measured with different H-donors.
(*a*) Total nitratase activities of the culture measured with formate (A) and lactate (B). C, growth curve.
(*b*) Nitratase activities of the cells measured with formate (A) and with lactate (B).

the amount of preformed enzyme with which the adapted cells had been endowed during their initial, previous adaptation.

It is also clear that, in the nitrate medium, adaptation of the previously unadapted cells proceeds very rapidly, and that the relatively higher nitratase activity of the adapted cells is not maintained for very long. After 4 hours the activities have become more or less equalized. The fall in the nitratase activity of adapted cells after 6 hours is probably due to the known toxic effect of the high concentration of nitrite and other nitrate reduction products which are formed ; continued incubation of the previously unadapted cells produces a similar fall, not shown in this experiment. The further adaptation of previously adapted cells when grown with nitrate is simply a reflection of the difficulty of achieving maximal adaptation merely by treatment of washed cells with glucose and nitrate without growth. Growth with fumarate gives a similar result to that with tetrathionate, the only difference being a greater initial production of " basal " nitratase in both adapted ano unadapted cells.

It might legitimately be objected that in determinations of activities of complex enzyme systems such as nitratase, different results might be obtained according to what H-donor was used. However, in an experiment illustrated by Fig. 5, nitratase activities of adapted cells have been followed during growth in the tetrathionate medium in parallel for a direct comparison between formate and lactate as H-donors. There is an unexplained drop in the total nitratase, measured with lactate, after 4½ hours, and there is a bigger fall in nitratase activity during the whole period when using lactate than when using formate. On the whole, however, the differences are relatively slight, and the same general picture is shown whichever H-donor is used for the nitratase assay.

DISCUSSION.

These results show :

(*a*) That " basal " nitratase formed by cells on growth without nitrate is subject to considerable variation.

(*b*) That preformed nitratase of adapted cells growing in the absence of nitrate remains apparently unmodified at least over a period during which cell mass has increased 8-fold.

Little comment is desirable on (*a*), especially since this will form the subject of further work at a later date. It seems likely that most adaptive enzymes are formed, at least to a slight extent, in the absence of substrate. Even in the case of yeast galactozymase—not normally detectable except after adaptation in the presence of galactose—Spiegelman and Reiner (1947) have shown that the enzyme can in fact increase under certain conditions in the absence of the substrate. The amount of basal enzyme formed varies from one strain of micro-organism to another, and might be expected to depend as well, to some extent, upon non-specific environmental factors.

With regard to (*b*), the inactivation of the nitratase system during washing and dilution of the cells has led to some inaccuracy, particularly during the early part of growth. Nevertheless, the results are clearly quite different from those that might be expected were some sort of interaction between adaptive enzymes, as reported by Spiegelman and Dunn (1947) for yeast galactozymase and maltozymase, occurring. Preformed nitratase remains apparently unmodified while the bacterial protoplasm increases, and the nitratase activity per cell approaches

the normal unadapted level. Still less is there any suggestion that treatment with nitrate has conferred on the cells some intrinsically increased ability to produce nitratase. The increase in total nitratase of the culture of adapted cells is no more than that which occurs with unadapted cells forming the usual amount of "basal" enzyme.

SUMMARY.

Nitratase activities of nitrate-adapted cells of the coliform bacterium "1433" growing in a medium without nitrate have been studied.

This preformed nitratase of adapted cells remains unmodified during growth of the rest of the bacterial protoplasm; so that the enzyme activity per cell gradually falls, during growth, to its normal preadaptive level, by a simple process of "dilution-out."

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THE "LEWIS" BLOOD GROUP CHARACTERS OF ERYTHROCYTES AND BODY-FLUIDS.

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A NEW blood group character designated "Lewis" was first described by Mourant (1946) who found that the red blood cells of about 25 per cent of English people were agglutinated by anti-Lewis sera and that the agglutination was independent of the ABO, MN, Rh, P, Lutheran and Kell systems. Jakobowicz, Simmons and Bryce (1947) described a further example of such a serum and Dr. Mourant informs us that he has had several sera of similar character sent to him from the Regional Blood Transfusion Laboratories in this country.

Andresen (1947) reported that he, and independently Dr. Freisleben, had encountered several sera which agglutinated the red cells of 21 per cent of adult Danes. It is now known that the antibody in these sera is identical with the antibody in Mourant's original sera. Andresen (1947) observed that his sera agglutinated the erythrocytes of about 70 per cent of Danish children who were less than 7 months old, and that parents, both of whose red cells failed to agglutinate with these sera could have children whose erythrocytes were agglutinated. He concluded that the agglutinability of the erythrocytes of adults behaves as a recessive Mendelian character, that is, adult persons whose red cells possess this agglutinability are genetically homozygous.

In a further paper Andresen (1948) described an additional antibody which in most of its reactions showed an antithetical relationship to the original Lewis antibody. The red cells of 6 per cent of Group O and about 37 per cent of Group A_1 persons, however, were not agglutinated by either of these sera. Andresen considered that the gene A_1 could have an epistatic action on the agglutinogen defined by this additional antibody.

A revised notation to cover the recent developments in the " Lewis " blood group system has recently been proposed by Andresen, Callender, Fisher, Grubb, Morgan, Mourant, Pickles and Race (1949) and has been used throughout the present paper. The two known Lewis genes recognized by Mourant and by Andresen respectively are now designated *Le*^{a} and *Le*^{b}, the corresponding antibodies as anti-Le^a and anti-Le^b and the gene products as Le^a-substance and Le^b-substance. If the antiserum, anti-Le^a, is used alone in agglutination tests the phenotype of the cells which react is defined as Le(a +), those not agglutinated, as Le(a −).

Our immediate interest in the Lewis blood group system arose from the observation that in a group of 62 persons all those whose red cells were agglutinated by anti-Le^a serum and who were presumably of the genotype Le^{a}Le^a, were nonsecretors of their corresponding A-, B-, H-substances. The results of the complete investigation, a preliminary account of which has been published by Grubb (1948), are now given.

EXPERIMENTAL.

Materials and Methods.

Test sera.

Anti-Le^a sera from nine persons, including the original serum from Mrs. Lewis, were used. Eight of the sera were obtained through the courtesy of Dr. Mourant, who had identified them as anti-Le^a sera. Two of these sera were from persons of Group AB. The anti-Le^a serum " Hughes," which was used throughout the work described in this paper and was kindly supplied by Miss Boorman, Miss Dodd and Mr. Gilbey, of the National Blood Transfusion Service, came from a person with the following group characters: A_2, Le(a −), secretor of A- and H-substances, CDe/CDe, Kell-ve, Lu(a −). This serum is referred to as the " Standard " anti-Le^a serum, and its anti-Le^a character was fully confirmed by us in tests involving a panel of known Le(a +) (9) and Le (a −) (27) bloods. A small amount of a serum " Buckstein " which was considered to be identical with Andresen's second type of anti-Lewis serum, now designated as Le^b antibody, was also available.

Test-cells.

Standard test-cells were Group O cells selected as representing the genotypes Le^{a}Le^a and Le^{a}Le^b. Cells considered to represent the genotype Le^{a}Le^a were strongly agglutinated by the standard serum, were not agglutinated by anti-Le^b and were obtained from a person whose saliva inhibited completely at a dilution of 1:100 the standard dose of anti-Le^a serum. Cells which were not agglutinated by anti-Le^a serum, were agglutinated by anti-Le^b serum and came from a person one parent of whom was of the phenotype Le(a +) were accepted as being heterozygous Le^{a}Le^b cells. The saliva of the heterozygous person showed some power to inhibit anti-Le^a agglutinins.

Agglutination and inhibition tests.

All agglutination and inhibition tests were carried out with once-washed, freshly-prepared erythrocyte suspensions. The degree of agglutination was observed microscopically after smearing a drop of the test material, serum-erythrocyte suspension, on a glass slide. Agglutinations were read after the tests had stood for at least 2 hours at 14–16°. It was observed that after washing the Le(a +) cells six times with saline they were agglutinated by the " standard " anti-Le^a serum to a much lower titre (4) than was normally observed with once-washed cells (16).

The influence of temperature on the degree of agglutination brought about by the " standard " anti-Le^a serum on Group O erythrocytes believed to represent cells of the genotypes Le^aLe^a, Le^aLe^b and of the phenotype Le(a — b —) was observed. The serum failed to agglutinate any of the cells at 37° but reacted weakly with Le^aLe^a cells at 25° and more strongly (titre 16) at 16°. The Le^aLe^b cells were not agglutinated at these temperatures but reacted weakly at 10°. Cells of the phenotype Le(a — b —) remained unagglutinated at 10°.

Owing to the strictly limited amount of the anti-Le^a and anti-Le^b sera available the agglutination technique described by Mourant (1948) for Rh agglutination was employed. The inhibition tests were carried out as follows: The serum (0·01 ml.) was filled into a series of tubes (5 × 50 mm.) by means of a Pasteur pipette graduated at 0·01 ml. and an equal volume of the appropriately-diluted test-fluid was added. The contents were mixed by gentle tapping and allowed to stand at 14–16° for 30 minutes. The erythrocyte suspension (0·01 ml. of a 1–2 per cent red cell suspension) was then added to each tube and the contents mixed. After standing for at least 2 hours at 14–16° the tubes were examined for agglutination.

The tests used to determine the presence or absence of A and B substances in the secretions and body-fluids were performed as described by Morgan and King (1943). The detection of the so-called " O " substance (the H-substance of Morgan and Watkins, 1948) was made by means of an anti-H serum engendered in the rabbit against an artificial antigen built up from purified H-substance and the conjugated protein of the O antigen of *Shigella shigae*. The H activity of the materials was also established by similar inhibition tests which used a serum from the eel *Anguilla vulgaris* (Jonsson, 1944; Grubb, 1949), which serum, at a dilution of 1:100, agglutinated O cells strongly but was without action on A_1B cells.

The pig stomach extracts examined were prepared by autolysis of individual stomach linings at pH 3–4 (Aminoff, Morgan and Watkins, 1946; Bendich, Kabat and Bezer, 1946; Chadwick, Smith, Annison and Morgan, 1949). The human ovarian cyst fluids were from women belonging to Groups A, B, O and AB. Fluids from secretors and nonsecretors within the ABO classification were investigated (Morgan and van Heyningen, 1944).

The preparations of A- and H-substances were obtained by methods already described (Morgan and King, 1943; King and Morgan, 1944; Morgan and Waddell, 1945) and purified until the materials were essentially homogeneous by physical, chemical and immunological tests. Full details for the preparation of these materials will be given elsewhere. The A-substance contained 5·7 per cent N and 9·1 per cent $CO.CH_3$, and showed a *dextro* rotation, $\frac{[\alpha] + 16° \pm 3}{5461}$. The examination of the products of hydrolysis with 0·5 N

HCl revealed that the A-substance contained 37–38 per cent hexosamine (as base) and gave rise to 56 per cent reducing sugars measured as glucose. The α-amino acid N, 2·1 per cent, comprised 38 per cent of the total N. The H-substance contained 5·4 per cent N and was *laevo*-rotatory [α]–30°. The material gave rise to 35 per cent hexosamine (as base) and 53 per cent reducing sugars after hydrolysis at 100° with 0·5 N HCl. A mucoid material, obtained from an ovarian cyst fluid which was devoid of A, B, H, Le^a or Le^b activity was used together with dextran, as a control substance, in inhibition tests. The mucoid contained 5·9 per cent N and showed no significant rotation.

RESULTS.

The examination of 212 erythrocyte specimens from nonrelated adults living in South-East England showed that 47 (22·2 per cent) of them were Le (a +). The salivas of the same persons were tested for the secretion of A-, B- and H-substances and the result showed that 49 (23·1 per cent) were non-secretors of these substances. There was some selection within the ABO classification and too few Group O persons were included.

A rather larger group of similar results representing all specimens examined is set out in Table I, from which it will be seen that of 222 individuals investigated, 57 were Le(a +) and nonsecretors of the A-, B- and H-substances. The remainder (165) were Le(a —) and of these 163 secreted the appropriate A-, B- and H-factors. In this group, therefore, there are two persons who are Le(a —) and nonsecretors of A-, B and H-substances.

TABLE I.—*Correlation Between Le^a Blood Groups and Secretor Character Within the ABO Classification.**

	O.	A_1.	A_2.	A.	B.	AB.	Total.
Erythrocytes Le(a +) A,B,H nonsecretors	23	11	6	10	5	2	57
Erythrocytes Le(a —) A,B,H secretors	47	45	14	30	18	9	163
Erythrocytes Le(a +) A,B,H secretors	0	0	0	0	0	0	0
Erythrocytes Le(a —) A,B,H nonsecretors	0	1	1	0	0	0	2
	70	57	21	40	23	11	222
			118				

* This table includes the results recorded earlier (Grubb, 1948).

The secretion of a water-soluble, serologically specific Le^a-substance was investigated by means of inhibition tests made with saliva at three dilutions (1:5, 1:25 and 1:100) using the standard anti-Le^a serum " Hughes," titre 1:16 and Le^aLe^a test-cells. The first 10 saliva specimens examined were investigated with two additional anti-Le^a sera using test cells obtained from four different Le (a +) persons. The only difference observed was that a weaker serum gave a higher inhibition titre, a result which could have been anticipated. A summary of the results obtained with salivas from 80 persons are set out in Table II, from

which it is evident that all persons so far investigated whose red cells are Le (a +) secrete Le^a-substance in the saliva, whereas only some of the persons whose red cells are Le(a −) do so. The salivas of persons from the former group are on the whole more potent than the active salivas obtained from the latter group. A specimen of gastric juice obtained from a person of phenotype Le (a +) contained Le^a-substance.

TABLE II.—*The Results of Inhibition Tests Using Saliva and Anti-Le^a Serum.*

Saliva from persons with	Number of specimens.	Percentage of salivas inhibiting anti-Le^a agglutination. Saliva dilution 1:5	1:25	1:100
Le(a −) red cells	50	84	40	0
Le(a +) red cells	30	100	100	90

The examination of the distribution of the water-soluble Le^a-substance in the body-fluids was extended to a large number of ovarian cyst fluids and the results of inhibition tests, which are set out in Table III, showed that some cyst fluids contained relatively large amounts of Le^a-substance. The cyst fluids were collected earlier for another purpose and it is, unfortunately, not known whether the red cells of the persons concerned were Le(a +) or Le(a −). It will be seen, however, that against a strongly-agglutinating dose of the "standard" serum some cyst fluids could be diluted many thousands of times, and were then able to inhibit completely the agglutinating action of the anti-Le^a serum. One cyst fluid, "UN", showed no significant amounts of A-, B- and H- substances but was rich in Le^a-substance. Preparations of purified and essentially homogenous human Group A-substance and H-substance of both human and animal origin show no significant power to inhibit the agglutination of Group O, Le (a +) erythrocytes by anti-Le^a serum.

TABLE III.—*Showing the Inhibition of Anti-Le^a Serum by Ovarian Cyst Fluids.*

Cyst fluid.	Group.	ABH Secretor (S) or non-secretor (s).	Dilution of cyst fluid inhibiting the agglutination of Le(a +) erythrocytes by anti-Le^a serum. 1:10	1:100	1:1000	1:3000	1:12,000	1:50,000
67	O	S	0	0	0	0	0	0
115	B	S	0	0	0	0	0	0
28	B	s	0	0	0	1	2	2
105	A	S	0	0	0	0	1	2
111	A_1B	S	0	0	1	2	3	3
117	A_1B	S	0	1	2	2	3	3
29	A_1B	s	3	3	3	3	3	3
UN	—	s	0	0	0	0	0	0
Saline	—	—	3	3	3	3	3	3

Degrees of agglutination: O, no agglutination; 1, a few groups of 2–3 cells; 2, larger groups with many free cells; 3, many small clumps.

The results of a few similar tests set up to detect the secretion of a soluble Le^b-substance are given in Table IV. A specimen of mucoid obtained from an

ovarian cyst fluid which showed Le^b activity inhibited a strongly agglutinating dose of anti-Le^b serum at a dilution of $1:2 \times 10^6$. It may be concluded, therefore, that this gene product is also secreted in a water-soluble form.

TABLE IV.—*Showing the Inhibition of Anti-Le^b-Serum by Saliva.*

Donor's red cell phenotype.	Final dilution of saliva inhibiting anti-Le^b serum. 1:3	1:15	1:75
Le(a +)	3	3	3
Le(a +)	3	3	3
Le(a − b +)	0	0	0
Le(a − b +)	0	0	0
Le(a − b −)	3	3	3
Le(a − b −)	3	3	3

Degrees of agglutination as in Table III.

Of 165 persons whose erythrocytes failed to react with anti-Le^a serum, 163 secreted the appropriate A, B, and H factors in a water-soluble form, whereas 2, whose blood belonged to the Groups A_1 and A_2 respectively, failed to do so. The reactions of the blood and saliva specimens from these individuals were confirmed on fresh samples and there appears to be little doubt that these persons are exceptions to the rule that individuals whose red cells are not agglutinated by anti-Le^a serum secrete the appropriate A-, B-, and H-substances.

The erythrocytes of one of the individuals (M.R., Group A_1) were not agglutinated by any of our panel of anti-Le^a or anti-Le^b sera and her saliva failed to inhibit the agglutination of A_2 cells by natural (human) anti-A serum or the haemolysis of sheep cells by rabbit anti-sheep cell serum. The saliva from these two individuals contained neither Le^a- nor Le^b-substance. The red cells of both persons were of the phenotype Le(a − b −).

MISCELLANEOUS OBSERVATIONS.

During the investigation a number of additional observations were made which seem worth recording. For example, no natural anti-Le^a agglutinins were detected in 21 normal rabbit or in 14 normal chicken sera. Specific Le^a-substance was not present in the mucoid materials prepared from 50 different pig stomach linings by acid autolysis and it would appear that preparations of A- and H-substances from this source can, therefore, be used for the neutralization of anti-A and anti-H agglutinins which may be present in human anti-Le^a sera. A mucoid material prepared from sheep submandibular gland (McCrea, unpublished) which showed considerable activity as a receptor substance for heated influenza virus likewise showed no Le^a activity. Anti-Le^a agglutinins were not absorbed by specimens of red cells from six rabbits and were not neutralized by the specific polysaccharide of Pneumococcus, Type XIV, or by the purified O antigen of *Shigella shigae*.

The serum from a person of genotype Le^aLe^a neutralized a strongly agglutinating dose of anti-Le^a serum although at a dilution of 1 : 2 only, whereas the serum from a person of the phenotype Le(a − b −) was without power to inhibit. Le^a-substance is, therefore, most probably present in low concentration in the serum of persons whose red cells are agglutinated by anti-Le^a serum.

The enzymic inactivation of the specific Le^a- and Le^b-substances.

The action of enzyme preparations obtained from culture filtrates of certain strains of *Cl. welchii* (Type B) on purified preparations of the Le^a-substance was examined. It had already been shown that these enzymic preparations inactivate serologically the A-, B- and H-substances (Morgan, 1946. Stack and Morgan, 1949) and that the enzymic activities could to some extent be differentiated by heating the enzyme preparation at 56° for 1 hour whereby the activity against the group substances A and B is lost. The heated solutions, however, are able to destroy the serological character of the H-substance. The Le^a-substance (1·0 ml. of 0·1 per cent solution in buffer at pH 6·4) was mixed with an equal volume of the enzyme solution and incubated at 37° for 2 hours in the presence of toluene. The enzyme solution used was (*a*) unheated, (*b*) heated at 56° for 1 hour, and (*c*) heated at 100° for 15 minutes. After incubation the enzyme-substrate solutions were heated at 100° for 10 min. to inactivate an enzyme present which brings about a sensitization of the red cells used in the agglutination-inhibition test performed subsequently to measure the degree of inactivation of the Le^a-substance. The results of an experiment of this kind are given in Table V from which it will be seen that the Le^a-substance is rapidly destroyed, whereas the enzymic material, after heating for 1 hour at 56° is almost inactive. Similar results were obtained with a partially purified specimen of Le^b-substance.

TABLE V.—*Showing the Inactivation of Le^a-Substance by a* Cl. welchii (*Type B*) *Enzyme Preparation.*

Treatment of enzyme.	Dilution (× 10^4) of Le^a-substance inhibiting the agglutination of O, Le(a +) cells by anti-Le^a serum.								
	1:2.	1:4.	1:8.	1:16.	1:32.	1:64.	1:128.	1:256.	1:512.
Unheated . .	0	0	1	2	3	3	3	3	3
Heated 56° 1 hr. .	0	0	0	0	0	1	2	3	3
„ 100° 15 min..	0	0	0	0	0	0	1	2	3

Degrees of agglutination as in Table III.

DISCUSSION.

A close correlation of the Lewis blood-group character with the secretor-nonsecretor status within the ABO classification has already been recorded by Grubb (1948) in a preliminary communication involving the data obtained from a relatively small group of adults. The results of the main investigation are communicated in this paper and confirm the earlier finding that persons whose red cells react with anti-Le^a serum are also nonsecretors of the A-, B- and H-substances. More recently, Race, Sanger, Lawler and Bertinshaw (1949) have likewise fully confirmed this interesting relationship. Furthermore, these authors showed that the data obtained from family studies are consistent with Andresen's view that adults whose red cells are agglutinated by anti-Le^a serum are genetically homozygous.

The frequency of bloods in our material giving agglutination with anti-Le^a serum, 22·2 per cent, agrees closely with the figure 22·7 per cent found by

Race *et al.* (1949) in a similar English population. The frequency of nonsecretors of A-, B- and H-substances, 23 per cent, found by us is of the same order as that reported for other populations. The corresponding figures are, for Germany, 22·0 per cent (Schiff), Denmark, 26·0 per cent (Hartmann, 1941) and New York, 18 per cent (Schiff see Wiener, 1946).

Le^a- and Le^b-substances are secreted in a water-soluble form as are the A-, B- and H-substances and, therefore, the terms secretor and nonsecretor should not be used without indicating clearly the group system involved. Le^a- and Le^b-substances resemble the A-, B- and H-substances in general physical and chemical properties. They are heat stable, water-soluble substances of mucoid nature and are readily decomposed by an enzyme in *Cl. welchii* (Type B) culture filtrates. The Le^a-substance occurs in saliva, gastric juice, ovarian cyst fluids and serum. The presence of the Le^b-substance has not been tested for in gastric juice or serum but it has been shown to occur in a water-soluble form in saliva and ovarian cyst fluids.

Le^a-substance was clearly demonstrable in the saliva from 30 persons whose red-cells were of the phenotype Le(a +). The majority of salivas from persons whose erythrocytes were Le(a −) also contained Le^a-substance. These observations are very different from those encountered in the ABO system where, for example, every fifth person belonging to Group A does not secrete A-substance, and A-substance does not occur in the secretions of persons whose red cells fail to react with anti-A serum, that is in those persons who belong to Groups O or B.

It seems as if all persons possessing the *Le^a* gene secrete Le^a-substance. One consequence of accepting Andresen's view that persons whose red cells are agglutinated by anti-Le^a serum are of the genotype Le^aLe^a, is that a majority of persons whose erythrocytes fail to react with anti-Le^a serum nevertheless possess an Le^a gene. It is not surprising, therefore, that the specific Le^a-substance may be encountered in the secretions of these persons. In secretions and body-fluids the Le^a-substance is in solution and its presence is demonstrated by means of the very sensitive agglutination-inhibition test. In red cells, on the other hand, the specific Le^a-substance is part of a fixed surface structure, the Le^a agglutinogen, and may be subject to the influence of co-existing heterologous determinant groupings and other unspecific factors which may combine to reduce the sensitivity of the agglutination test. The differences in substrata and technique may, therefore, conceivably make the whole difference between detection (in the saliva) and non-detection (in the erythrocytes) of a single dose of the Le^a-gene. Although it may now be accepted that the Le^a-substance occurs in the secretions of many persons whose red cells seem to lack the corresponding agglutinogen, it can be anticipated that the practical application of this knowledge, the demonstration of the presence of a single dose of the *Le^a* gene by means of inhibition tests using saliva, will be beset with difficulties. It is known that within the ABO system the differentiation of secretors from nonsecretors is in certain instances not very satisfactory (Hartmann, 1941) and the results of the present study indicate that the Le^a secretion-nonsecretion phenomenon represents more gradual and continuous variation than is met with in ABH secretion-nonsecretion. It is a matter of conjecture, therefore, whether some of the cases are to be classified as secretors or as nonsecretors of Le^a-substance.

As the great majority of salivas obtained from persons whose red cells fail to react with anti-Le^a serum contain some specific Le^a-substance, only a minority

of salivas could be used for the neutralization of anti-A or anti-B agglutinins in anti-Le^a sera.

In our material all persons who possess Schiff's secretor gene, *S*, have red cells which are not agglutinated by anti-Le^a serum. There is, therefore, a close although inverse relationship between the Le^a and *S* genes. It is known from Andresen's results, however, that the majority of these bloods will react with anti-Le^b serum and, furthermore, it is probable that no bloods of the phenotype Le (a + b +) exist. It might be anticipated, therefore, that there is some kind of relationship between Le^b and *S* genes. Owing to the very limited amount of anti-Le^bserum available we have been forced to confine our tests to a few instances where the erythrocytes or secretions were deemed of special interest. It was thought earlier that a very close relationship might exist between H-substance and Le^b-substance and in consequence a number of specimens of H-substance of human origin were tested for their capacity to neutralize H and Le^b antibody and the two activities expressed as a ratio. The results showed that H-substance and Le^b-substance cannot be identical. Furthermore, we have encountered an individual of the phenotype O, Le(a — b —) who secretes H-substance but not Le^a or Le^b-substances. Dr. Race informs us that he has encountered three persons with similar characters. The frequency of the occurrence of the phenotype Le(a — b —), secretor of A-, B- and H-substances, cannot be estimated on our material as the very limited amount of anti-Le^b serum available has precluded an adequate investigation for the presence of this gene product.

From what is already known of the Lewis system it can be surmised that Le^b-substance and some or all of the A-, B-, H-substances will be frequently found together in the body-fluids and secretions.

It is evident from the results given in Table I and from the findings of Race *et al.* (1949) that the correlation between Le(a +) blood group character and secretor status within the ABO classification, is a very close one. On the basis of probability it would seem impossible that these results have arisen by chance. A simple rule seems to hold and may be expressed as: Adult persons with Le(a +) red cells are nonsecretors of A-, B- and H-substances and adult persons who possess Le(a —) red-cells are secretors of these substances. As a result of this relationship it follows that in adults a good estimate as to the secretor-nonsecretor status of an individual within the ABO classification can be made on the basis of a simple agglutination test with anti-Le^a serum. So far less than 1 per cent of individuals are known to be exceptions.

Individuals whose red cells are agglutinated by anti-Le^a serum most probably possess the Le^a-gene in double dose and they are nonsecretors of the A-, B-, H-substances. Schiff and Sasaki (1932) have shown that persons who are nonsecretors of the A-, B-, H-substances possess the gene *s* in double dose. Does the close correlation of Le(a +) red cell character and non-secretion of the A-, B-, H-substances indicate that the genes Le^a and *s* are identical? We have observed a few individuals of the phenotype Le(a — b —) in whose saliva no A-, B-, H-, Le^a- or Le^b-substances were demonstrable. The absence of these gene products is not due to impaired health or old age and indeed the examination of 10 persons, within the age group 55–80, who were suffering from Addisonian anaemia and were thus nonsecretors of hydrochloric acid, revealed no exceptions to the rule that Le(a —) persons are secretors of the appropriate A-, B-, H-substance. It seems probable, therefore, that the phenotype Le

(a — b —) nonsecretor of A-, B-, H-substances is an accurate reflection of the genetical constitution of these individuals and the existence of such individuals makes it improbable that the genes Le^a and s are identical. Is this an example of linkage in man? If Le^a and s genes are linked they must be very closely linked, otherwise crossing-over would have obliterated the Le(a +)-nonsecretor association in the general population.

If the genes Le^a and s are linked, this linkage is so close that one might place these genes, and consequently the genes Le^b and S, in one genetic system. Furthermore, the general similarity of the physical and chemical properties of the soluble products of the Le^a, Le^b and S genes might be an additional reason for considering that they belong to the same system.

The Lewis system and its relationship to the secretor-nonsecretor phenomenon within the ABO classification cannot be discussed at length in this paper. It is, nevertheless, worth considering briefly. It is suggested that there are three allelomorphic pairs of genes, S and s, Le^a and Le^α, Le^b and Le^β, which occupy contiguous loci. The genes Le^α and Le^β, are allelomorphic to the genes Le^a and Le^b respectively and are unknown at present. The symbols Le^α and Le^β are used here solely to illustrate the hypothesis. Such a system would be similar in many respects to the Rh system and of the eight possible combinations on a single chromosome, four only, $s\ Le^a\ Le^\beta$, $s\ Le^\alpha\ Le^\beta$, $S\ Le^\alpha\ Le^\beta$, $S\ Le^\alpha\ Le^b$, occur at all frequently in our material. The data so far recorded would appear to fit a system of this kind which comprises a closely-packed constellation of three individual genes. We are well aware of the tentative character of the hypothesis. The predictions inherent in the scheme, however, are largely open to experimental proof.

SUMMARY.

1. The examination of blood and saliva of 212 persons has shown that 22·2 per cent are Le(a +) and that these individuals all fail to secrete A-, B- and H-substances. There is, therefore, a very close association between Le (a +) red cell character and inability to secrete in terms of the A, B and H blood-group factors.

2. Le^a- and Le^b-substances are present in secretions of persons of the appropriate genotype and show the same general physical and chemical characters as the A-, B- and H-substances.

3. It seems probable that all persons possessing the Le^a gene secrete Le^a-substance.

4. Some human ovarian cyst fluids are a convenient and potent source of the Le^a- and Le^b-substances.

5. The specific Le^a- and Le^b-substances are inactivated by an enzyme present in *Cl. welchii* (Type B) culture filtrates.

6. Le^b- and H-substances are not identical

7. It is suggested that the Le^a, Le^b, S and s genes belong to one and the same system

8. Among 222 individuals, two nonsecretors of A-, B-, H-substances whose red cells were Le(a —) were found These two individuals were also non-secretors of Le^a- and Le^b-substances and their red cells were Le(b—) A person of the phenotype O, Le(a — b—), who was a secretor of H-substance and a nonsecretor of Le^a- and Le^b-substances was also encountered The occurrence

of persons with these serological characters might be considered to indicate the existence in the Lewis system of additional, unknown genes.

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ALVEIN.

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DURING a survey of the antibacterial properties of aerobic spore-forming bacilli, Gilliver (1949) found that a strain of *Bacillus alvei* (code number 642 in the collection of Dr. Gibson of Edinburgh), when streaked on lemco agar, produced diffusible substances that inhibited the growth of a number of Gram-positive and Gram-negative bacteria. This paper contains a preliminary account of one antibiotic, named alvein, which is formed by *Bacillus alvei* 642 in certain liquid media.

Production.

The production and purification of alvein were followed by the use of the cylinder plate method of assay (Heatley, 1944). *Staph. aureus* (N.C.T.C. 6571) was used as a test organism, and a dry preparation of alvein hydrochloride was adopted as an arbitrary standard. At a dilution of 1 in 10,000 the standard preparation produced inhibition zones of from 15 to 18 mm. in diameter.

Early attempts to produce the antibiotic by growing *B. alvei* in stationary liquid culture met with little success. Active material was formed in a lemco-peptone broth, but only when a surface growth of the bacillus was facilitated by some mechanical support, such as by pads of cotton wool floating in the medium. The use of stationary cultures was abandoned when it was found that an antibiotic was readily formed by deep fermentation in a medium containing glucose, corn steep liquor and inorganic salts.

Medium and conditions of culture.

The organism was grown in deep aerated culture in the following medium:

$NaNO_3$	3·0 g.	$FeSO_4$	0·01 g.
KH_2PO_4	1·0 g.	Corn steep liquor	10 ml.
KCl	0·5 g.	Glucose	10 g.
$MgSO_4\ 7H_2O$	0·5 g.		

made up to 1 litre with tap water and adjusted to pH 7·0 before autoclaving.

The fermentation was carried out in 10 litre bottles containing 8 litres of medium. One ml. of sterile triamyl citrate per litre of medium was added, after autoclaving, to prevent frothing. The inoculum was 200 ml. of an aerated overnight culture in the same medium. The bottles were placed in a water bath at 37° C. and the cultures aerated through perforated glass bulbs at the rate of about 1 litre of air per litre of culture fluid per minute. After from 40 to 48

hours the culture fluid was at pH 6·6–7·0 and its activity was approximately equivalent to that of a 1 in 80,000 solution of the standard alvein hydrochloride.

In a medium containing lower concentrations of corn steep liquor and glucose the activity fell off rapidly after reaching its maximum. When higher concentrations of corn steep liquor and glucose were used, a large proportion of the activity appeared to be due to an antibiotic other than alvein, which was active against *Myco. phlei* but not against *Staph. aureus*.

Extraction and purification.

In early small-scale experiments the antibiotic was extracted directly from the culture fluid (adjusted to pH 9·0) by butanol. For dealing with larger quantities of fluid it was more convenient to use a process involving adsorption onto charcoal. The process was carried out in the following manner.

The culture fluid was cleared, either by standing overnight or by centrifuging. The supernatant liquor was brought to pH 7, and shaken with 0·5 per cent charcoal (Farnell grade 14 neutral) which adsorbed the active material. The latter was eluted by shaking the charcoal with a two-phase mixture of n-butanol and N/5 HCl (1:3), the total amount used being 1/10th of the volume of the original culture fluid. Most of the eluate was concentrated in the butanol layer. It was transferred to aqueous solution by mixing the butanol with one volume of ether, shaking the mixture with one volume of distilled water, and adjusting the pH to 2·0. The resulting aqueous solution was neutralized and filtered.

A two-phase system of butanol and dilute HCl had previously proved useful for eluting the antibiotic ayfivin from charcoal (Arriagada, Abraham, Sharp, Savage, Heatley and Sharp, 1949). Butanol is adsorbed on the charcoal and displaces the antibiotic, which partly enters the aqueous phase. In the case of alvein the antibiotic then passes largely into the layer of remaining butanol, the final system consisting of three phases. The method presumably depends on the reduction in concentration of the desorbed substance in the eluting solvent which results from the entry of the substance into the aqueous phase.

Steenberg (1941) first described a procedure for desorption from charcoal which involved alternate treatments of the charcoal with an organic solvent, such as benzene, and water. The adsorbed substance was insoluble in the organic solvent, but was displaced by it from the charcoal and could then be removed by water. The use of benzene and an aqueous phase was not successful with ayfivin. The efficiency of butanol and dilute HCl for eluting ayfivin, laterosporin (Barnes, 1949) and alvein may be associated with the significant solubility of these antibiotics in butanol.

After extraction from butanol, alvein was precipitated from aqueous solution by the addition of an excess of saturated picric acid at 4° C. The picrate was dried in a vacuum desiccator and then converted to the hydrochloride by stirring with ethyl alcohol containing 5 per cent N HCl. Alvein hydrochloride was precipitated from the acid-alcohol extracts by the addition of an excess of dry ether. The precipitate was washed with ether and dried *in vacuo*. The yield was about 3–4 mg. per litre of culture fluid and represented a recovery of 20–30 per cent of that present in the original culture fluid.

The properties of alvein which are recorded in the following sections refer to material obtained in this manner.

Further purification.

To obtain some idea of the homogeneity of this preparation an eight tube counter-current distribution (Craig, Golumbic, Mighton and Titus, 1945) was carried out with 20 mg. of active material. Isobutanol-phenol (1:1) and 0·1 N aqueous acetic acid were used as the two phases and the total volume of solvents in each tube was 2 ml. The overall partition coefficient of the antibacterial activity in the system was approximately one. After the distribution, Tube O contained 6·5 per cent of the material, but no activity, while Tube 8 contained 5·6 per cent of the material and about the same proportion of activity. The amounts of material and activity in Tubes 2–7 approximated to those expected if these tubes contained a single active substance with a partition coefficient of 1·28. It was thus possible that the preparation contained less than 10 per cent of inactive material and that the major portion of the activity was associated with a single substance, although a second active substance was present in a proportion of about 5 per cent.

Chemical Properties.

The preparation of alvein hydrochloride gave the following values on elementary analysis: C, 49·0; H, 8·7; N, 12·9; S, 1·2; Cl, 8·9.

The hydrochloride was easily soluble in ethanol and in water, but insoluble in acetone, chloroform or ether. A solution in water had a pH of about 3·8. As the pH was raised by the addition of sodium hydroxide the substance became much less soluble. Precipitation began when the pH approached 7 and increased as it was raised further to 9. The precipitate dissolved on addition of ethanol.

On shaking a solution of alvein hydrochloride in aqueous alcohol with a suspension of silver oxide, or on adding an amount of sodium hydroxide equivalent to the chloride present, the solution became alkaline to phenolphthalein, indicating that the free substance was a strong base.

Alvein was extracted by butanol from solution in water at pH 9. It was not removed by butanol from a solution in water at pH 3, but was readily extracted by phenol. The presence of small amounts of sodium chloride greatly affected the partition coefficient of alvein between butanol and an aqueous solution at pH 3. One per cent sodium chloride caused most of the antibiotic to enter the butanol phase, and 0·1 per cent sodium chloride had a significant effect. This action of salt was presumably responsible for the fact that after elution from charcoal under acid conditions the antibiotic was found largely in the butanol layer.

In aqueous solution at room temperature alvein lost no measurable activity in two hours at pH 2, 7 or 9, but its activity was reduced in 0·1 N or 0·5 N HCl. It retained its activity for one hour in boiling aqueous solution at pH 2, but in a similar solution at pH 9 half its activity was lost. The activity of an aqueous solution of alvein (0·2 mg. per ml.) was reduced to about one eighth of its original value after incubation for 4 hours with commercial trypsin (1 mg. per ml.) at pH 8. Incubation with 50 per cent horse serum resulted in very little loss in activity.

Alvein gave a positive Sakaguchi reaction for arginine, but negative colour reactions for tyrosine and tryptophane. After hydrolysis for 20 hours with 6 N HCl at 105° C., the product gave a strong ninhydrin reaction. The colour density yielded by a given weight of alvein hydrochloride, determined by the

method of Moore and Stein (1948), was almost identical with that given by the same weight of leucine. Analysis of the hydrolysate by paper chromatography (Consden, Gordon and Martin, 1944) indicated the presence of cystine, lysine, serine, threonine, alanine, valine and leucine. Lysine and leucine appeared to be present in large amounts. There was no indication of the presence of glutamic acid or aspartic acid.

The preparation thus behaved as though it consisted mainly of a strongly basic polypeptide.

Biological Activity.

Antibacterial activity.

The activity of alvein against a number of bacteria was determined by a serial dilution method in nutrient broth. The solutions of alvein hydrochloride were sterilized by filtration through a sintered glass bacteriological filter, and diluted in a two-fold series. Ten per cent of serum was present in the tests with *Str. pyogenes* and *C. diphtheriae*. The tubes were inoculated from overnight broth cultures of the test organisms, and the results were read after overnight incubation, the end-point being taken as that concentration of alvein which completely prevented visible growth. Further incubation for one day did not decrease the inhibitory level more than two-fold.

The test with *Myco. tuberculosis* was kindly performed by Dr. A. Q. Wells. The organism was cultured in Dubos' medium containing 10-fold dilutions of alvein, the inoculum being 0·1 ml. of a 7-day culture in Dubos' medium. The results were read at 4 days and 11 days.

The results obtained are shown in Table I.

TABLE I.—*Antibacterial Activity.*

Test organism.	End point at 24 hours: 1 part alvein hydrochloride in x thousands.
Staph. aureus	512
Str. pyogenes (C.N. 10)	128
C. diphtheriae var. *gravis*	128
C. xerosis	8,000
Bact. coli	32
Ps. pyocyanea	16
B. anthracis	512
Myco. phlei	32,000
Myco. tuberculosis (P.N.)	100 (4 days) 10 (11 days)

Attempts to increase the resistance of *Staph. aureus* by growing it in the presence of sub-inhibitory amounts of alvein were unsuccessful. The action of alvein on *Myco. tuberculosis* appeared to be bactericidal.

Haemolytic activity.—Alvein is haemolytic. Its action on human erythrocytes at 30° C. was measured quantitatively in a photoelectric apparatus designed by Dr. W. E. van Heyningen, to whom we are indebted for collaboration. At a dilution of 1 in 5,000, alvein produced 78 per cent haemolysis of a 0·4 per cent suspension of human red cells in 10 seconds. At 1 in 15,000 it produced 22 per cent haemolysis under the same conditions. After the first rapid change, subsequent haemolysis was relatively very slow.

Bernheimer (1947) found that tyrocidine, in contrast to certain bacterial haemolysins, caused a rapid haemolysis which was followed by a slow haemolysis at a constant rate. The phenomenon appeared to be due to inhibition of the action of tyrocidine by the products of haemolysis. Although the haemolytic action of alvein resembles that of tyrocidine the two substances are quite distinct in other properties.

Other Antibacterial Substances Produced by B. alvei, 642.

At least one antibacterial substance other than alvein appeared to be produced by *B. alvei* in Czapek-Dox broth. The evidence for an additional substance depends on the following observations :

(1) When samples of culture fluid were incubated with commercial trypsin, their activity against *Staph. aureus* and *C. xerosis* was reduced to 1/8th–1/16th of the original value. This reduction could be accounted for by the effect of trypsin on the alvein present in the fluid. The activity against *Myco. phlei*, however, was reduced only to one half.

(2) The recovery of activity from the culture fluid was only about 10 per cent. when *Myco. phlei* was used as test organism, whereas it was 20–30 per cent when *Staph. aureus* was used.

It thus appeared that there was a substance in the culture fluid which was active mainly against *Myco. phlei* and was not readily inactivated by trypsin, but which was lost during the extraction of alvein. The antibacterial activity of the culture fluids produced by deep fermentation in Czapek-Dox broth containing high concentrations of corn steep liquor and glucose was probably due mainly to the presence of this substance.

SUMMARY.

An antibiotic named alvein has been extracted from the culture fluid of a strain of *Bacillus alvei*. Alvein has the properties of a strongly basic polypeptide. It is highly active against a variety of Gram-positive bacteria and less active against certain Gram-negative bacteria. It is haemolytic.

We wish to thank Miss M. Bond for doing some of the serial dilution tests, and Miss Ann Pill and Mr. D. Jackson for technical assistance. One of us (K. G.) is indebted to the Agricultural Research Council for personal grants and for grants towards the expenses of this work.

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THE ANTIBACTERIAL PROPERTIES OF SOME SPECIES OF AËROBIC SPORE-FORMING BACILLI.

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In recent years some 20 apparently distinct antibiotics have been obtained from the culture fluids of various species of aërobic spore-forming bacilli. A small number of them are capable of protecting animals against experimental infections with bacteria susceptible to their action. Some have been used in man, particularly for local application on infected surfaces.

This paper records the results of a survey of the antibacterial activity of identified strains of bacilli. The object of the survey was to select strains, if possible not previously investigated, as a preliminary to more detailed investigation of their antibacterial properties.

EXPERIMENTAL.

Most of the cultures were obtained from the National Collection of Type Cultures, or from the collection of Dr. T. Gibson, of Edinburgh, and the identity of all strains was established or checked by the methods of Gibson (1944) and Gibson and Topping (1938). To keep the scope of the work within practicable limits the strains were examined by a restricted number of somewhat arbitrary procedures. The procedures were designed to detect those strains which secreted antibacterial substances, and to differentiate as far as possible between different substances. Strains were selected for further study if they produced large zones of inhibition in any of the tests, and if the antibiotics concerned appeared to possess interesting antibacterial ranges. It was also considered advantageous if the substances were not readily inactivated in the presence of trypsin or serum.

Detection of active strains.

The production of antibacterial substances at 37° C. on one solid medium and in one liquid medium was studied.

Solid medium.—The solid medium was a lemco agar containing 1 per cent peptone, 1 per cent lemco and 0·5 per cent sodium chloride, all the strains being tested on this medium by the cross-streak method. Primary streaks of bacilli were incubated for 24 hours, and the test organisms were then streaked at right angles to them. After a further 24 hours' incubation measurements were taken of any inhibitions. The following were used as test organisms:

Staph. aureus N.C.T.C. 6571, *Bact. coli*, *Ps. pyocyanea*, *S. enteritidis* Gaertner, *S. typhi*, *C. xerosis*, *C. diphtheriae* var. *gravis*, *Myco. phlei* (laboratory strain; N.C.T.C. strains 525 and 1471) and *Myco. smegmatis*.

Liquid medium.—Potato dextrose broth was chosen as the liquid medium since it had already been found to be suitable for the production of bacilysin by a strain of *B. subtilis* and of ayfivin by a strain of *B. licheniformis* (Arriagada, Abraham, Sharp, Savage, Sharp and Heatley, 1949). In making a litre of broth, 200 g. of diced potatoes were steamed in 500 ml. of tap water for one hour. After the bulk of the debris had settled the liquor was strained through cotton-wool, the volume made up to 1 litre with tap water, and 10 g. of dextrose added. The bacilli were grown in layers of broth about 1 cm. deep in 500 ml. Erlenmeyer flasks. The activity of the culture fluids was assayed daily for 12 days by the cylinder plate method (Heatley, 1944), using *Staph. aureus* and *Myco. phlei* as test organisms. The antibacterial content of the cells was not investigated.

Differentiation between the antibacterial substances produced.

Some differentiation between the substances secreted on the cross streak plates was possible on the basis of the differences in antibacterial ranges.

Several methods were used in attempts to differentiate between the substances secreted into the liquid medium.

(*a*) Culture filtrates were sterilized by gradocol filtration, and incorporated with agar in gutters cut along the diameter of lemco agar plates. After diffusion overnight at 4° C. these were cross streaked with a range of bacterial test organisms, and inhibitions were measured after overnight incubation at 37° C.

(*b*) Samples of culture filtrates were incubated for 6 hours, either with 1 mg. per ml. of commercial trypsin, or in 50 per cent horse serum, and the residual activity was then measured by the cylinder plate method.

(*c*) Many of the zones of inhibition of *Staph. aureus* produced by the culture fluids of *B. subtilis* and *B. pumilus* contained scattered colonies of resistant staphylococci (Abraham, Callow and Gilliver, 1946). Plates seeded with staphylococci resistant to one culture filtrate were used for testing other culture filtrates, in the hope that the specificity of the resistant organisms would yield information of value in grouping these antibiotics.

RESULTS.

Active strains.

The results are summarized in Table I.

In all, 165 strains of bacilli were tested, and of these, 61 strains belonged to the *Bacillus subtilis* group, comprising *B. subtilis*, *B. pumilus* and *B. licheniformis*. All the strains of this group were active against one or more of the test organisms on one or both media, only one strain failing to secrete active substances into lemco agar and five into potato dextrose broth. Strains of other species of bacilli were considerably less active, only about one-third showing activity on the solid medium, and only two strains, both of *B. cereus*, secreting active substances into potato dextrose broth. The species showing activity on lemco agar were *B. brevis*, *B. alvei*, *B. laterosporus*, *B. circulans*, *B. megatherium* and *B. polymyxa*.

The importance of pellicle formation.

The secretion of antibacterial substances into potato dextrose broth by strains of the *B. subtilis* group was correlated with their ability to grow in the form of a

pellicle. All the consistently active strains formed thick pellicles on potato dextrose broth and the five inactive strains failed to form any surface growth. Five strains varied from time to time both in their ability to form pellicles and to secrete antibacterial substances, and the two characteristics were closely connected. Cultures of those strains which had formed thin pellicles and secreted active substances were not appreciably more turbid when shaken than entirely submerged cultures of the same strain which were inactive.

Differentiation between the antibiotics produced.

Solid medium.—It had been hoped that it would be possible to classify the antibiotics produced in agar into several groups by the differences in antibacterial range shown on the cross-streak plates. Upon repetition of these tests, however, it was shown that for most strains the inhibition of *Staph. aureus* and of the Gram-negative test organisms was erratic, and that the only criteria sufficiently reliable for this purpose were the relative inhibition of the various strains of *Myco. phlei*, and the relative inhibition of *C. xerosis* and the laboratory strain of *Myco. phlei*. The actual figures obtained with any strain varied from day to day, but the relative inhibition of these test organisms was unchanged. Zones of inhibition of greater width than 15 mm. were rarely recorded.

Two main groups were found :

Group 1.—The zone of inhibition of *Myco. phlei* N.C.T.C. 1471 was greater than or equal to that of the laboratory strain, and the zone of inhibition of the N.C.T.C. 525 strain was appreciable. The zone of inhibition of *C. xerosis* was also equal to or greater than that of the laboratory strain of *Myco. phlei*.

Group 2.—The zone of inhibition of Strain 1471 was less than that of the laboratory strain, the difference being more than 3 mm. The inhibition of Strain 525 was slight, and that of *C. xerosis* was less than that of the laboratory strain, the difference being again more than 3 mm.

Most of the results fell into either of these two groups, only a small number of intermediate types remaining.

Among the *B. subtilis* group, all the strains of *B. licheniformis* exhibited the Group 1 type of antibacterial range, and the strains of *B. subtilis* and *B. pumilus* in general gave the Group 2 results, a few showing either Group 1 or intermediate results. The same two types of antibacterial range were found among the other species of bacilli, different strains of the same species sometimes showing different pictures.

Liquid medium.—The results obtained with the substances secreted in potato dextrose broth are summarized in Table I. Specific points are discussed in the following paragraphs.

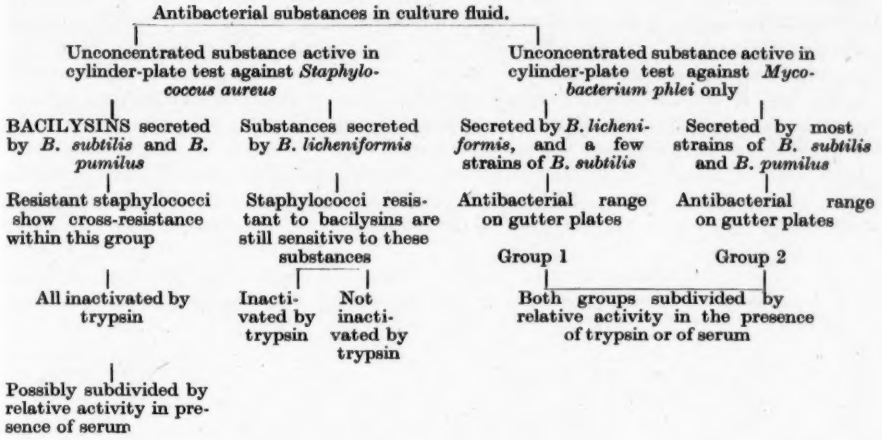
The active substance secreted into potato dextrose broth were of two main types. The first inhibited the growth of *Staph. aureus* in the cylinder plate tests, and the second inhibited the growth of *Myco. phlei*. The two types of substances were secreted independently of each other in the same culture fluids.

The evidence for this distinction was as follows :

(1) The culture fluids of some strains which were usually active against both test organisms were occasionally active only against one of them.

(2) In some instances the peak activity against one test organism was attained before that against the other.

TABLE I.


Antibacterial substances in culture fluid.
Unconcentrated substance active in cylinder-plate test against Staphylococcus aureus
Unconcentrated substance active in cylinder-plate test against Mycobacterium phlei only
BACILYSINS secreted by B. subtilis and B. pumilus
Substances secreted by B. licheniformis
Secreted by B. licheniformis, and a few strains of B. subtilis
Secreted by most strains of B. subtilis and B. pumilus
Resistant staphylococci show cross-resistance within this group
Staphylococci resistant to bacilysins are still sensitive to these substances
Antibacterial range on gutter plates
Antibacterial range on gutter plates
Group 1
Group 2
All inactivated by trypsin
Inactivated by trypsin
Not inactivated by trypsin
Both groups subdivided by relative activity in the presence of trypsin or of serum
Possibly subdivided by relative activity in presence of serum

(3) The effect of incubation with trypsin upon the culture fluids of many strains was to remove all the inhibitory action against *Staph. aureus*, while that against *Myco. phlei* remained unchanged or was somewhat reduced.

Of the substances inhibitory against *Staph. aureus*, those secreted by strains of *B. subtilis* and *B. pumilus* were provisionally called bacilysins, since they were apparently indistinguishable from each other and from a substance secreted by a strain of *B. subtilis*, N.C.T.C. 7197, to which this name had originally been given (Abraham, Gilliver and Florey, 1947, unpublished). The bacilysins were inactivated by incubation with trypsin and were insoluble in organic solvents. Staphylococci resistant to the bacilysin secreted by one strain of *B. subtilis* were also resistant to those secreted by other strains. They were, however, still sensitive to the substances active against *Staph. aureus* which were secreted by strains of *B. licheniformis*. The latter substances therefore appeared to be distinct from the bacilysins.

The substances active against *Myco. phlei* in the cylinder plate tests appeared to be responsible for the two types of antibacterial range that occurred when culture fluids were tested by the gutter plate method. This was shown by the fact that when the bacilysin content of culture fluids was destroyed by incubation with trypsin, the inhibitions on gutter plates remained unchanged except for a reduction in the activity against *Staph. aureus*. Moreover the failure to distinguish between the bacilysins by the very sensitive method of tests with resistant staphylococci showed that these substances were unlikely to exhibit differences in antibacterial range.

The differentiation on the gutter plates between the substances active in cylinder plate tests against *Myco. phlei* depended, as in the cross-streak plates, upon the relative inhibition by them of *C. xerosis* and *Myco. phlei*. In Group 1 the inhibition of *C. xerosis* was greater than or equal to that of *Myco. phlei* (laboratory strain), and in Group 2 the inhibition of *Myco. phlei* was considerably greater than that of *C. xerosis*, which frequently was not inhibited at all.

The Group 1 picture was characteristic of the substances secreted by *B. licheniformis*, whereas those secreted by *B. subtilis* and *B. pumilus* usually gave the Group 2 picture. A few strains of *B. subtilis* showed the Group 1 range. There were no intermediate types of result.

The substances secreted by *B. cereus* were inactive against *Staph. aureus* when tested by the cylinder-plate method, but remained unclassified by the gutter plate method since they were apparently inactivated by gradocol filtration. The activity in these culture fluids was increased, however, by incubation with trypsin, and this distinguished them from the culture fluids of all other species.

TABLE II.

Species.	Number tested.	Active in potato dextrose broth.	Activity on gutter plates. Number of strains. Group		Activity on cross-streak plates. Number of strains. Group			
			1	2	1	2	Intermediate.	Inactive.
B. subtilis	36	35	8	27	1	24	9	1
B. licheniformis	18	18	18	—	17	—	1	—
B. pumilus	7	3	—	3	1	1	4	1
B. cereus	16	2	—	—	1	7	3	5
B. brevis	13	—	—	—	5	—	5	3
B. laterosporus	5	—	—	—	2	1	1	1
B. alvei	9	—	—	—	6	1	—	2
B. circulans	11	—	—	—	1	2	—	8
B. megatherium	13	—	—	—	—	1	—	12
B. polymyxa	2	—	—	—	—	1	—	1
B. fusiformis	10	—	—	—	—	—	—	10
B. carotarum	9	—	—	—	—	—	—	9
B. mycoides	8	—	—	—	—	—	—	8
B. macerans	6	—	—	—	—	—	—	6
B. coagulans	3	—	—	—	—	—	—	3
B. firmus	1	—	—	—	—	—	—	1

Selection of strains for further investigation.

The following strains were selected for further study :

B. subtilis A 14.
B. alvei Gibson strains 642, 949, 1067.
B. laterosporus Gibson strains 1066, 1080.
B. brevis N.C.T.C. 5402, Gibson 442.
B. circulans N.C.T.C. 6351.
B. polymyxa N.C.T.C. 4747.

The strain of *B. subtilis* was selected on the grounds of its high activity in potato dextrose broth. Although the zones of inhibition produced by the culture fluids of most strains of *B. subtilis* rarely reached 30 mm. in diameter, the culture fluid of this strain contained substances which caused zones of inhibition of 30–35 mm. in diameter and 22–25 mm. in diameter respectively on plates seeded with *Myco. phlei* and *Staph. aureus*. The activity against *Myco. phlei* was only slightly reduced by incubation either with trypsin or with serum. The antibacterial substances secreted by this strain have been investigated in some detail (Newton, to be published).

The other strains showed on cross streak plates appreciable inhibition of the Gram-negative bacteria in addition to a high activity against the Gram-positive

bacteria, and tests on blood agar showed that these effects were not due to the presence of hydrogen peroxide.

Investigations have been made of the substances secreted by one strain each of *B. laterosporus* and *B. alvei* (Barnes, 1949; Gilliver, Holmes and Abraham, 1949).

DISCUSSION.

It is recognized that the restriction of the number of tests employed imposed certain limitations upon the survey. The methods of assay, for example, detected only those substances that were diffusible in agar, and it is also possible that different results would have been obtained by incubating cultures at several different temperatures and in several different media. Lemco agar was a more successful choice than potato dextrose broth in the latter respect, since many of the bacilli other than the *B. subtilis* group secreted active substances when grown on it. It is unlikely, however, that any one liquid medium would have been useful for all the species, and the use of potato dextrose broth appeared to be justified at least in the case of the *B. subtilis* group. The choice of test organisms might also be said to have been somewhat restricted. Nevertheless it is thought likely that a fairly good idea of the antibacterial possibilities of the bacilli was obtained.

It seems clear that the substances secreted on the same medium by all strains of *B. licheniformis* were similar to each other in antibacterial range, and distinct from the corresponding substances secreted by most strains of *B. subtilis* and *B. pumilus*. Further distinctions between these groups of substances appeared to necessitate the use of methods outside the scope of this survey. The occurrence of intermediate types of result on cross streak plates, for example, suggested that there was a range of substances being secreted, those at the two extremes falling into the arbitrarily defined Groups 1 and 2. The groups themselves each consisted therefore of a collection of substances with a broadly similar antibacterial range, but not necessarily with any other property in common. An experiment in which both primary streaks and test organisms were bacilli with the Group 1 antibacterial range demonstrated clear-cut differences of range within the group; but an extension of this experiment was unfortunately impracticable.

The demonstration of the importance of pellicle formation in the production of antibiotics by the *B. subtilis* group is of some interest. One effect of the formation of a surface pellicle is that the improved aëration permits of an increased growth of the organism, and the secretion of detectable amounts of antibacterial substances under these conditions may be a direct consequence of this. Observations made with submerged cultures of *B. subtilis* strain A 14 and with surface cultures of *B. alvei* G 642 tend to support this view. The secretion of antibacterial substances by these bacilli was increased or modified when growth was stimulated, either by more efficient aëration methods in the first instance, or by the support of a surface growth of the latter organism by mechanical means (Newton, unpublished; Gilliver *et al.*, 1949).

The phenomena exhibited by the five variable strains of *B. subtilis* and *B. pumilus* could not be explained in this manner. Nor, in the case of the strain of *B. alvei* referred to above, was aëration and consequent increased growth the decisive factor, since media were developed on which thick pellicles were formed

but into which no antibiotic was secreted. In the former case the secretion of detectable amounts of antibiotics which followed the establishment of a surface growth seemed to result from an increased or modified metabolic activity of the cells already present, rather than from a continued activity of an increased number of cells. In the case of *B. alvei*, factors such as carbon and nitrogen sources, growth factor requirements, temperature and hydrogen ion concentration were probably playing a part in limiting antibacterial activity.

The exact relationship between the substances demonstrated in this survey and those already described in the literature is not certain. The group of substances secreted in potato dextrose broth and inhibitory towards *Myco. phlei* probably comprises many different types, those secreted by the two strains of *B. cereus* presenting certain features of interest. Of the strains selected as the result of the cross streak tests, the two so far investigated, *B. alvei* and *B. laterosporus*, have been found to secrete in certain liquid media substances distinct from those already described (Gilliver *et al.*, 1949; Barnes, 1949). While it is by no means certain that these organisms secreted the same substances into both the liquid medium and the solid medium, it would appear likely that an investigation of other strains selected by the cross-streak method would be worth while.

SUMMARY.

One hundred and sixty-five strains of aërobic spore-forming bacilli were tested for their ability to secrete antibacterial substances into lemco agar and into potato dextrose broth. All the strains of the *B. subtilis* group were found to be active in one or both media. Only one-third of the strains of other species showed any activity, and this was confined to lemco agar. It was found possible to group the substances produced on the basis of their antibacterial ranges, and by the effect of trypsin and serum upon them. Certain strains have been selected for more detailed investigation.

I am indebted to Sir Howard Florey and to Dr. E. P. Abraham for their interest and advice throughout this work, and to Dr. Gibson for his help with the identification and taxonomy of the bacilli. I am grateful also to Miss A. M. Holmes for help with many of the experiments, and to Miss E. Page and Mr. D. Jackson for technical assistance. To the Agricultural Research Council I offer my thanks for personal grants and for grants for materials and equipment.

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SEROLOGICAL DIAGNOSIS IN MUMPS.

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HABEL'S discovery in 1945 that mumps virus can be cultivated in chick embryos (Habel, 1945) has made laboratory work with mumps possible on a larger scale. It also makes serological diagnosis possible. The serological diagnosis is of special interest in clinically doubtful cases and in patients with encephalitis of unknown etiology, a certain number of which are due to mumps virus (Kane and Enders, 1945).

The purpose of this paper is to investigate the reliability of complement fixation and haemagglutination inhibition tests as diagnostic methods in mumps.

MATERIAL.

Sera were collected from 60 patients admitted for mumps in the hospital for infectious diseases in Stockholm. All patients had mumps with classical symptoms. In the material neither doubtful cases nor cases with encephalitis without symptoms from the salivary glands are included. Sera were taken as early as possible during the illness and subsequently every tenth day.

The material is not representative of mumps in general. It is selected according to the indications for hospitalization. The rate of complications, especially encephalitis and orchitis, is higher than usual in mumps.

Sera from 51 adults, who had not had mumps during the last two years, were used as a control material.

The strain used was kindly supplied by Dr. G. Henle in Philadelphia. In this paper it is referred to as the A-strain. This strain was used throughout for the experiments unless otherwise stated. For cross-inhibition tests another three strains were used. Two of them, the WAL and STO strains, were forwarded by Miss Patricia Lind in Melbourne. The third one, the V strain, was isolated in this laboratory during an epidemic of mumps in 1947–48. The sera were collected during the same epidemic.

The V strain was isolated in the amniotic cavity. Saliva was taken on the second day of illness, was mixed with 1500 units of penicillin and 1000 μg. of streptomycin per ml. and injected in an amount of 0·2 ml. into the amniotic cavity. The saliva had been kept at −70° C. for about a month before being tested. The strain showed agglutination of guinea-pig and chicken red cells from the first passage. In spite of a great number of allantoic passages it could not be adapted to the allantoic cavity.

METHODS.

Cultivation of virus.

The A strain was maintained through allantoic passages in eggs 7 days old, using infected allantoic fluid in a dilution of 10^{-2} as inoculum. The allantoic fluid was harvested after 6 days' incubation of the eggs at 35° C. Under these conditions the best yield of virus was obtained. The strain grew irregularly in the allantoic cavity, the logarithmic haemagglutination titres ranging from not measurable to 3·01 and seldom higher. Thus it was necessary to test the harvested fluid from each egg for the presence of virus.

Complement-fixation test.

To a series of 0·2 ml. twofold serum dilutions from 1/4 to 1/512 0·2 ml. of antigen and 0·2 ml. of complement were added. The mixture was incubated in a waterbath at 37° C. for 45 minutes. After addition of 0·5 ml. of sensitized sheep red cells the tubes were left in the waterbath for another 30 minutes and then placed in a cold room at 4° C. overnight. The test was read the following morning.

Antigen. – An infected allantoic fluid was used, which had been diluted 1/2 to 1/8 according to a preceding antigen titration against a standard serum. It was kept frozen at −16° C. until used. Merthiolate 1/10,000 was used as preservative.

Complement.—Guinea-pig complement was titrated in the presence of the same amount of antigen as used in the test. The amount of complement in the last tube with complete haemolysis was taken as a unit. In the test 1·5 units were used. The complement was stored at −16° C. and discarded if more than one week old.

Sensitized sheep red cells.—Red cells were washed three times and used in a dilution of 0·75 per cent. Amboceptor was added to the red cell suspension in an amount corresponding to 4 units per tube. The mixture of red cells and amboceptor was allowed to stand for 15 minutes before being used.

Serum.—All sera were inactivated at 56° C. for 30 minutes. Sera from the same patient were titrated simultaneously with the same antigen and the same complement dilutions. They were kept frozen at −16° C.

Controls.—(1) One tube containing all reagents except antigen. (2) One tube in which normal allantoic fluid was substituted for antigen. (3) Control with negative serum. (4) Control with positive serum.

The titres are expressed throughout as the negative log of the initial serum dilution, that is the serum dilution before antigen, complement and sheep red cells have been added. The calculated 50 per cent haemolysis limit was taken as endpoint of the complement-fixing capacity.

The agglutination titres of the virus suspensions were determined according to Salk (1944).

Haemagglutination inhibition test.

Salk's modification of Hirst's technique was used throughout (Salk, 1944). The sera were thus diluted in 0·5 ml. of 0·25 per cent chicken red cell suspension and titrated with the same amount of a virus suspension containing 4 units of virus, the amount of virus causing a ± reaction in haemagglutination titration

taken as a unit. The tubes were left at room temperature for 1½ hours and then placed at 4° C. overnight. The test was read the following morning by observing the bottom patterns.

In 1948 Hirst reported that the normal inhibitor of serum could be destroyed by treatment of the serum with sodium periodate. This report indicated a way to investigate whether the lack of correlation between haemagglutination inhibition and complement-fixation titres (see below) might depend upon the disturbing effect of the normal inhibitor. In investigating this possibility, treatment with periodate was performed in the following way: 0·2 ml. of serum was mixed with 0·2 ml. of 0·05 M $NaIO_4$ and was allowed to stand for 2 hours. The excess of periodate was then neutralized by the addition of 0·8 ml. of 5 per cent glucose.

Each serum was titrated simultaneously with and without treatment with periodate.

Sera from the same patient were titrated at the same time. They were inactivated at 56° C. for 30 minutes.

The titres assigned to the sera are expressed as the negative log of the final serum dilution after addition of all reagents. The tube with a ± reaction, or if there were more than one, the first one of them, was taken as endpoint of the inhibiting capacity. If there was no tube with ± reaction the endpoint was calculated through interpolation between the last tube with no agglutination and the first tube with total agglutination.

The reading was sometimes considerably hampered by the fact that the limit between agglutination and inhibition was not sharp enough. The endpoint of some sera was repeatedly so diffuse that no satisfactory titre could be determined and the serum had to be excluded from the investigation.

EXPERIMENTAL.

Reproducibility of complement-fixation, haemagglutination and haemagglutination inhibition titres.

In order to assess the reproducibility of the titres a number of titrations with each of the methods were carried out. In the haemagglutination inhibition and complement-fixation titrations a known positive serum with a high complement-fixation and inhibition titre was used. Each method was tested in the course of one day and with the same dilutions of the participating reagents throughout the experiment

Complement-fixation test.—The greatest difference between two titres of a total of 50 was 0·24. The standard deviation (σ) was 0·056, the standard deviation of σ 0·0056 and thus $3\sqrt{2}\cdot\sigma = 0{\cdot}24$. Accordingly a difference corresponding to one tube, that is 0·30, may be considered significant.

The result is presented in Table I.

Haemagglutination titration.—The precision of haemagglutination titres was studied by 59 haemagglutination titrations of the same virus suspension. The standard deviation was found to be 0·11 the standard deviation of σ 0·0101 and $3\sqrt{2}\cdot\sigma$ 0·47 (Table I). The greatest difference between two titres was 0·38.

Haemagglutination inhibition test.—40 titrations of the same serum were carried out. The greatest difference between two titres was 0·45 and all except

two lay within the range 3·16 and 3·46. The standard deviation was 0·120, the standard deviation of σ0·0134 and $3\sqrt{2\cdot\sigma} = 0{\cdot}51$. With the same level of significance as for the complement-fixation test a difference corresponding to 2 tubes, that is 0·60, may be considered significant. The result is presented in Table I.

TABLE I.—*Deviations and Standard Deviation of Complement-fixation, Haemagglutination and Haemagglutination Inhibition Test.*

	Number of titrations.	Mean of titres.	Deviation from mean. Greatest.	Deviation from mean. Least.	Standard deviation σ.	Standard deviation of $\sigma\left(\frac{\sigma}{\sqrt{2n}}\right)$	$3\sqrt{2\cdot\sigma}$.
Complement-fixation test	50	1·71	0·14	0·01	0·056	0·0056	0·24
Haemagglutination test	59	2·57	0·22	0·01	0·11	0·0101	0·47
Haemagglutination inhibition test	40	3·31	0·30	0·15	0·120	0·0134	0·51

If haemagglutination inhibition titres are determined at different times there are two more variable factors introduced into the test, namely, different virus and different red cell suspensions.

The effect of variation of the amount of virus was studied by titration of a known positive serum with a high inhibition titre with 4–512 units of virus. The result is presented in Fig. 1.

It is evident that there exists a linear relationship between log amount of virus and log inhibition titre. A fourfold increase of the amount of virus corresponds to a twofold decrease of serum titre. The inhibition titres are thus influenced by variation of the amount of virus to a comparatively slight extent. A similar relation between the amount of influenza virus and the corresponding mouse neutralization titre was earlier found by Horsfall (1939).

The haemagglutination titres may differ by 0·47 (see above) when the titrations are made on the same day and with the same dilutions of the reagents. This variation of the virus concentrations of the dilutions used for antibody titrations may cause a difference of 0·24 of inhibition titres, thus increasing the variations of inhibition titres to 0·75. A still greater difference of inhibition titres, 0·90, was found in duplicate titrations of 26 sera on different occasions. This difference might be explained by the use of different red cell suspensions.

In investigating the effect of variation of red cell suspensions it was found that titrations with red cells from different hens gave surprisingly great differences of titres. Differences of 1·5 and 1·8 were not uncommon. This is in agreement with the report of Anderson, Burnet and Stone (1946) that non-specific inhibitory titres showed a great variation when they were determined with red cells from different fowls, as well as Svedmyr's finding that titration of the inhibiting effect of normal allantoic fluid gave different results when red cells from different fowls were used (Svedmyr, personal communication).

The greatest variation of the means of the haemagglutination inhibition titres of 22 sera, tested with 4 different kinds of red cells, was 1·05 (Table II). Eight were acute phase and 14 convalescent sera. There was no certain difference between acute phase and convalescent sera concerning the influence of different

red cells on the inhibition titres (Table II). The maximal difference of titre values of the same serum among these 22 sera was 2·10 (7 tubes).

TABLE II.—*Means of Haemagglutination Inhibition Titres of Acute Phase and Convalescent Sera not Treated with Periodate and Titrated with Red Cells from 4 Fowls.*

	Fowl number: 1.	2.	3.	4.
Acute phase sera (8). .	1·94	2·00	2·95	2·86
Convalescent sera (14). .	2·00	2·10	2·95	3·13
Total (22). . . .	1·95	2·07	2·96	3·00

After treatment with periodate, 14 sera, 9 of which were convalescent and the others acute phase sera, were titrated with red cells from 4 fowls. The greatest difference of titre of the same serum was 1·50 (5 tubes). The mean of the titres,

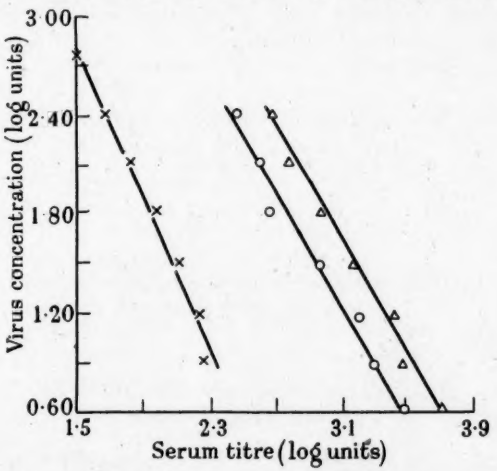

FIG. 1.—Correlation between haemagglutination inhibition titre of serum and virus concentration.
× Serum no. 175, no periodate.
△ „ „ 180, „ „
○ „ „ 181, periodate.

determined with red cells from the same fowl, was calculated. These calculated means varied from 1·59 to 2·17 (Table III). The corresponding figures for acute phase and convalescent sera were respectively 1·41 to 1·95 and 1·84 to 2·43 (Table III). Thus the variations of the titres of convalescent and acute phase sera were of about the same magnitude.

TABLE III.—*Means of Haemagglutination Inhibition Titres of Acute Phase and Convalescent Sera, Treated with Periodate and Titrated with Red Cells from Different Fowls.*

	Fowl number: 1.	2.	3.	4.
Acute phase sera (5). .	1·41	1·38	1·95	1·87
Convalescent sera (9). .	1·84	1·82	2·43	2·34
Total (14) . . .	1·70	1·59	2·14	2·17

In general the differences were smaller when the sera were treated with periodate before being titrated. Nevertheless, they were great enough to cause misinterpretations, if titres determined at different times were compared, and they were easily demonstrable both in acute phase and in convalescent sera.

Each virus suspension which was used for antibody titrations was titrated with the 4 different kinds of red cells, used for inhibition titrations. The variations lay within the distribution of haemagglutination titres, calculated above.

Control material.

Complement-fixation test.—None of the 51 sera contained enough antibodies to cause complete fixation in a dilution of 1:4. Six of them showed partial fixation. The remaining 45 sera showed no complement-fixing capacity under the conditions of the experiment.

Haemagglutination inhibition test.—The control sera inhibited in fairly high dilution the haemagglutination by mumps virus. As seen from Table IV, 32 of a total of 51 had an inhibition titre of 2·71 or more.

TABLE IV.—*Haemagglutination Inhibition Titres of Control Sera not Treated with Periodate.*

	Number of cases.								
	0.	1.	6.	10.	1.	25.	1.	7.	0.
Titre in log units .	<1·81	1·81	2·11	2·41	2·56	2·71	2·86	3·01	>3·01

Treatment of the sera with periodate strongly reduced the inhibition titres. No titre exceeded 2·28; 10 sera had a titre of 1·68 or more and 30 titres were lower than 1·38 (Table V).

TABLE V.—*Haemagglutination Inhibition Titres of Control Sera Treated with Periodate.*

	Number of cases.							
	30.	7.	3.	6.	1.	2.	1.	0.
Titre in log units .	<1·38	1·38	1·53	1·68	1·83	1·98	2·28	>2·28

Thus the control material showed a divergence between complement-fixation and inhibition titres which—as will be seen later—exists also between complement-fixation and inhibition titres of convalescent sera.

Comparative titrations of acute phase and convalescent sera.

The material consisted of 60 patients with clinical symptoms of mumps. In some of the cases sera were not obtainable early enough in the acute stage or late enough during convalescence. They are, nevertheless, presented in the table.

For every serum three titre values are presented, viz. complement-fixation titre and haemagglutination inhibition titre, determined with and without periodate.

Complement-fixation.—A significant rise of complement-fixing antibodies occurred in 47 patients (Table VI). In two cases no antibodies could be

demonstrated with the technique used, although sera were available up to two months after the onset of illness. In the remaining 11 cases a sufficient number of blood specimens could not be obtained. A patient was not considered as negative, unless negative specimens were obtainable during acute stage and subsequently up to 40 days after the onset of illness.

Haemagglutination inhibition test.—Titration of sera not treated with periodate revealed a significant rise of antibodies in only 11 cases. After treatment of the sera with periodate a significant rise could be demonstrated in 27 patients (Table VI).

Summing up the results, 80 per cent of the cases could be diagnosed with the aid of complement-fixation technique, and 18 per cent with the haemagglutination inhibition test. Treatment of the sera with periodate augmented the rate of positive cases in inhibition titrations to 45 per cent (Table VI).

The above data provide no absolute values of the reliability of the three serological methods, as in some cases a sufficient number of blood specimens was not obtainable. However, they may be used for comparison of the reliability of the methods.

TABLE VI.—*Titrations of Sera From* 60 *Mumps Patients.*

	Complement-fixation test.	Haemagglutination inhibition test.	Haemagglutination inhibition test. Periodate.
Number of positive cases	47	11	27
Per cent positive cases	80	18	45

If only those cases are considered in which a significant rise of antibodies could be demonstrated with one of the tests, a comparison between the three methods gives the following result: Complement-fixation test 94 per cent positive cases, haemagglutination inhibition test 26 per cent, and haemagglutination inhibition test, after treatment of the sera with periodate, 57 per cent.

This result indicates a great divergence between complement-fixation and inhibition titres of the whole material.

It is illustrated in Fig. 2 and 3.

Fig. 2 illustrates the differences of the rise of the titres. In this figure the mean of the individual increase of the respective titres, from the acute phase to 10 to 20, and 20 to 30 days after onset of illness, is reproduced. It can be seen that the average increase of haemagglutination inhibition titres was about 0·60 (2 tubes), when the titres were determined after treatment of the sera with periodate, but only 0·30 (1 tube) when they were determined without treatment with periodate. The average rise of complement-fixation titres was 1·5 (5 tubes).

From Fig. 3, in which the number of cases is plotted against the rise of titres, it is evident that the treatment with periodate brings about a closer correlation between complement-fixation and haemagglutination inhibition titres. On the other hand, it is also evident that the treatment with periodate does not make the divergence between the two methods disappear.

In may cases there was a significant rise of complement-fixation titre, but no rise of the inhibition titre. Two cases showed a significant rise of haemagglutination inhibition, but no rise of complement-fixation titre and in many cases the rise of the respective titres did not appear simultaneously.

Cross-inhibition titrations with 4 different strains.

The lack of correlation between clinical findings and the haemagglutination inhibition titres might be explained by differences between the strain used for titrations and the strain which caused the actual outbreak of mumps, especially

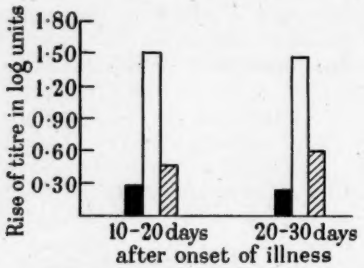

FIG. 2.—Average increase in log. units of complement-fixation and haemagglutination titres in 60 mumps patients.
■ Haemagglutination inhibition test.
□ Complement fixation test.
≡ Haemagglutination inhibition test. Periodate.

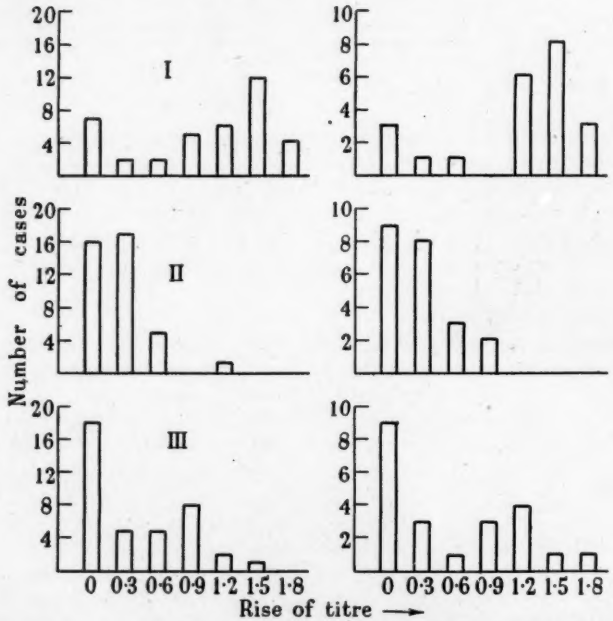

FIG. 3.—Distribution of the observed maximal increase of complement-fixation and haemagglutination inhibition titres. Material comprises 39 patients observed 10–20 days, and 20 patients observed 20–30 days after onset of illness.
I. Complement-fixation test.
II. Haemagglutination inhibition test.
III. Haemagglutination inhibition test. Periodate.

as the strain used throughout this investigation was isolated in the U.S.A., and the sera were collected from patients living in Sweden. Considering this possibility a number of cross-inhibition tests with 4 different strains were carried out. Only the A strain being adapted to the allantoic cavity, all the titrations used for comparison of different strains were performed with infected amniotic fluid.

Eighteen convalescent sera were titrated. The difference of the titres of these sera did not exceed 0·60 if they were not treated with periodate ; this is within the normal variation of titres determined with different virus suspensions. None of the strains showed any tendency towards constantly higher or lower titres than the others.

Titration of the same sera after treatment with periodate gave a somewhat different result. Twelve of 18 sera gave lower titres with the A strain than with the others (Table VII). Whether this is really due to strain differences is not possible to decide.

TABLE VII.—*Cross-inhibition Test with 4 Strains against Human Convalescent Sera, Treated with Periodate.*

Serum.	Virus strain. A.	V.	WAL.	STO.
1	1·38	1·38	1·38	1·38
2	2·58	2·58	2·58	2·58
3	1·38	2·89	2·58	2·58
4	2·28	3·19	2·89	2·89
5	1·98	3·19	2·89	2·89
6	1·68	1·98	1·98	1·98
7	1·68	2·58	2·58	2·58
8	2·58	2·58	2·28	2·28
9	2·58	3·19	3·19	2·89
10	2·89	2·28	2·58	2·28
11	2·28	2·58	2·58	2·58
12	3·49	3·19	2·58	3·19
13	1·68	1·68	1·68	1·68
14	1·98	3·19	3·49	2·89
15	1·38	2·28	2·58	1·68
16	1·38	2·58	2·58	1·98
17	2·58	3·19	2·89	3·19
18	1·68	2·89	2·58	2·28

Titration with homologous strain.

Titration of a number of sera against the V strain, which was isolated from a patient out of the outbreak of mumps, during which the sera were collected, showed the same divergence between complement-fixation and haemagglutination inhibition titres. Of 19 patients, 1 showed a rise of inhibition titre of 0·90, and 6 a rise of 0·60. Treatment of the sera with periodate revealed a significant rise of inhibition titres in 3 patients. In all except 1, complement-fixing antibodies could be demonstrated in significant amounts.

DISCUSSION.

On account of the high haemagglutination inhibition titre values of the control material it is evident that no conclusions as to infection with mumps virus can be drawn from a single haemagglutination inhibition titre. The control material is too limited to allow any conclusions whether the diagnosis can be established with the aid of a single complement-fixation titre.

The haemagglutination inhibition reaction is extensively used, both for experimental and practical purposes, especially in work with influenza virus. In spite of this fact, the experience and the knowledge of the reaction is still too limited to allow a definite judgment about its specificity and its value.

The data presented indicate that the haemagglutination inhibition reaction has a very limited value in serological work with mumps, both in its original and in its modified form. Evidently, it failed to reflect the actual antibody content of the sera in the material presented.

The great differences of haemagglutination inhibition titres which are determined with red cells from different fowls, emphasize the fact that inhibition titres should not be compared unless they are determined with the same red cell suspension. The causes of these great variations are still obscure. They do not occur only in titrations of "normal" sera, but also in sera containing specific inhibiting antibodies, and they do not disappear after treatment of the serum with periodate. If Hirst's hypothesis, that sodium periodate destroys the normal inhibitor of serum, is correct, the differences of titres should, thus not at least entirely, be depending upon variations of the action of the normal inhibitor. The factor responsible for the differences is probably to be found in the serum, not in the virus or red cells, as the haemagglutination by mumps virus was not influenced by different red cells. A possible explanation of the phenomenon is that the sera contained two or more inhibiting factors, one of which was destroyed by periodate.

The divergence between the complement-fixation and the haemagglutination inhibition titres could possibly be explained by the hypothesis that the sera tested contain antibodies which do not fix complement. However, the lack of correlation between clinical findings and inhibition titres seems to exclude such a possiblity.

It is not very likely that strain differences are responsible for the divergence of the titres. Comparative titrations with 4 different strains did not indicate any certain strain differences, and titrations of some of the sera with the virus strain causing the actual outbreak of the disease did not alter the results.

Reports of experiences with the haemagglutination inhibition test in mumps are still very scarce, and there is no investigation (on mumps) with which the results could be compared. However, Hoyle and Fairbrother, in 1947, reported that the complement-fixation test gave more clear cut results, and higher incidence of positive reactions in influenza, than the haemagglutination inhibition reaction. Stuart-Harris and Miller (1947) interpreted some peculiar and contradicting results with the inhibition test in influenza as depending upon the fact that the strains were newly isolated. It is possible that the inhibition reaction works better in titrations with influenza virus. In any case, as far as mumps is concerned, the reaction needs further investigation as to its specificity before it can be applied for practical serological work.

The complement fixation reaction yielded results which agreed with the clinical findings in all except two cases. This is in agreement with other reports of the test in mumps (Enders, Cohen and Kane, 1945).

SUMMARY.

In a material consisting of 60 mumps patients the complement-fixation test yielded results in agreement with clinical findings in all except two cases.

In 49 mumps patients a significant rise of antibodies could be demonstrated: in 94 per cent with the aid of complement-fixation technique, in 26 per cent with haemagglutination inhibition test, and in 57 per cent with haemagglutination inhibition test after treatment of the sera with periodate. Haemagglutination inhibition titrations of the same serum with red cells from different hens gave variations of the logarithmic titres up to 2·10 (7 tubes).

Cross-inhibition tests with 4 strains, one of which was isolated during the actual outbreak of the disease, did not reveal any certain strain differences.

It is concluded that the haemagglutination inhibition test failed to reflect the actual antibody content of the sera of the material presented.

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AN ELECTROPHORETIC STUDY OF THE COMPONENTS OF THE SERUM PROTEINS IN CIRRHOSIS OF THE LIVER.

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THE fundamental importance of the liver in protein metabolism has been firmly established by the experimental studies of Mann and his collaborators (Bollman and Mann, 1936), coupled with extensive studies of Myers and Keefer (1935), and Tumen and Bockhus (1937) on the circulating proteins in patients suffering from long standing damage to the liver parenchyma.

In 1929, Abrami and Wallich in France, and Salvesen in Scandinavia, using the salting out techniques then available, demonstrated the marked alteration in the ratio of albumin to globulin in the circulating proteins of patients, associated with chronic liver damage. In the years that followed, their principal findings were confirmed by other workers.

The utilization of the improved technique for electrophoresis, introduced by Tiselius (1939), offered a convenient means of making a more exact study of the changes in the circulating protein pattern associated with chronic progressive liver damage. Leutscher (1940) reported isolated analyses on two cases and followed this (1941) by single analyses of two additional cases. Further analyses

were published by Gray and Barron in 1943, and by Thorn, Armstrong and Davenport in 1946.

Incidental analyses of sera from patients suffering from chronic liver damage are recorded by Kabat, Hanger, Moore and Landow (1943) in a study of the cephalin flocculation test, and by Malmross and Blix (1946) in a study of the erythrocyte sedimentation rate.

CLINICAL MATERIAL.

An examination of available data suggested that it would be useful to investigate sera from patients suffering from chronic progressive liver damage in whom there was no other primary condition likely to effect the analysis. For this reason, cases of carcinoma of the bile ducts, carcinoma of the liver, haemachromatosis, and other primary conditions with which degenerative changes of the liver may be associated, were excluded. All patients have been followed for at least two years or until death. Diagnoses have been substantiated in five instances by complete autopsy and in two by laparotomy. Not less than two analyses have been carried out in every case. Certain ancillary biochemical investigations performed simultaneously are listed in Table I. Patients were afebrile when serum was collected for analysis.

METHODS.

Blood was drawn from patients while fasting and the serum dialyzed for 72 hours against three changes of phosphate buffer of ionic strength 0·2 and pH 8·0. The analyses were carried out in the Tiselius apparatus at 0·8° C. The concentration of protein in the solution actually submitted to electrophoresis was adjusted to 2·0 per cent $\pm$ 0·2 per cent in all analyses.

Cholesterol estimations were carried out by a modification of the method of Schoenheimer and Sperry (1934).

Normal levels by the method used tallied closely with the careful analyses carried out by Gardner and Gainsborough (1930). The thymol turbidity tests were carried out by the technique of Maclagan (1944*a*). Alkaline phosphatase levels are given in King units, the technique followed being that of King and Armstrong (1934). The bilirubin levels were estimated by the method of King, Haselwood and DeLory (1937).

RESULTS.

The results are shown in Table I. Of the observed patients, No. 2, 5 and 9 gave histories of attacks of infective hepatitis 4 years, 3 years and 6 years previously, others in the series gave equivocal accounts of ill health which might have been attacks of infective hepatitis. It was considered wiser to disregard these. Total protein levels vary from 4·0 to 8·5 g. per 100 ml. serum ; analyses 2*b* and 6*b*, both obtained on the day of death, show totals of 4·0 and 7·6 g. per 100 ml. serum respectively. It is evident that the total protein levels may bear little relation to the severity of the disease. In every case there was a gross decrease in albumin level but it was noticeable that the degree of ascites and general oedema did not correspond regularly with the albumin level. Thus, in spite of an albumin level falling to 2·0 g. per 100 ml., Case 9 showed no ascites nor was oedema demonstrable even when the patient was ambulant. Only

TABLE I.

No.	Sex.	Age.	Total protein g./100 ml.	Albumin. g./100 ml.	Globulin g. %. α_1	α_2	β	γ	Bilirubin mg. %.
1	Male	55	7·9	2·6	0·6	0·7	1·2	2·8	4·0
			7·7	2·7	0·4	0·7	1·1	2·8	3·0
2	„	46	4·8	1·6	0·45	0·4	0·9	1·45	2·5
			4·0	1·1	0·4	0·4	0·8	1·3	3·0
3	„	56	6·6	2·05	0·6	0·6	1·2	2·0	2·0
			6·0	2·0	0·5	0·5	1·1	1·9	3·0
4	Female	45	7·3	2·5	0·75		1·0	3·0	1·75
			8·5	2·9	0·8		1·5	3·5	1·0
5	„	55	6·4	2·5	0·6		0·8	2·4	1·5
			5·6	2·3	0·2	0·3	0·7	2·1	1·5
6	„	56	8·0	1·5	0·25	0·4	1·05	4·8	3·0
			7·6	1·4	0·2	0·3	0·9	4·7	..
7	Male	42	7·0	2·2	0·90		1·2	2·6	1·5
			8·0	2·4	0·90		1·6	3·0	0·4
8	Female	65	4·7	1·8	0·35		0·9	1·6	1·0
			4·8	1·9	0·4		0·7	1·8	1·5
9	„	64	8·2	2·0	0·3	0·3	0·8	4·8	2·0
			8·0	2·0	0·2	0·25	1·1	4·3	0·9
Normal			7·0	4·0	0·3	0·4	0·7	0·7	0
			—7·5	—4·5	—0·5	—0·6	—1·2	—1·0	—0·75

No.	Thymol turbidity units.	Alkaline Phosphatase units.	Total Cholesterol. mg./100 ml.	Approximate Duration from original diagnosis.	Ascites.
1	8	..	..	2 years	0
	..	..	..	2 years and 1 month	0
2	8	..	190	2 years	+
	8	..	..	2 years and 14 days (Died day of second analysis)	+
3	..	..	200	3 years	+
	8	..	..	3 years and 2 months (Died 3 months later)	+
4	8	20	..	2 years	slight oedema
	10	24	170	2 years 6 weeks	slight oedema
5	14	8	180	1 year	++
	14	6	..	1 year and 2 months (Died 3 weeks later)	++
6	9	16	190	1 year	+
	10	..	..	1 year and 7 days (Died day of second analysis)	+
7	12	16	..	1 year and 10 months	0
	8	11	170	2 years	0
8	8	12	155	Probably at least 5 years	++
	8	..	165	2 months later	++
9	14	6	170	1 year and 6 months	0
	16	9	180	1 year and 7 months	0
Normal	0	3	150		
	—4	—12	—220		

The total protein concentration is calculated from Kjeldahl estimation. The albumin and globulin concentrations are calculated from electrophoretic analysis. The low values for α- and β-globulins justify this procedure.

two analyses out of 18 showed β-globulin concentrations above the normal limits though in each case the total cholesterol in the serum was normal. For simplicity the α-globulins have been considered together. In 18 analyses, α-globulin was above normal limits in two, and below normal limits in 8, of the remaining eight, five fell below the mean of normal.

The circulating bilirubin levels were for the most part above normal levels though not of the degree usually associated with obstruction. The thymol turpidity levels were well above normal in every case. They showed little tendency to fluctuation, nor did their relative levels bear close relation to the patients' general condition. Thus Patient 9 with a thymol turbidity of 16 units was anxious to return home, while Patient 2 died with a thymol level of 8 units.

In those patients in whom observations on the level of alkaline phosphatase in the serum were carried out no gross increases were observed. The highest recorded was 24 in Patient 4. Maclagan (1944*b*) records that 6 out of 14 cases of cirrhosis studied by him had alkaline phosphatase levels between 14 and 24. The levels of serum cholesterol were all within normal limits.

DISCUSSION.

The precautions taken in selecting cases for study in the series have already been stressed. Previous workers have included in their studies patients suffering from carcinoma of the bile duct, cholelithiasis with stone in the common duct, and xanthomatosis.

The lack of correlation between the total protein levels and clinical severity has already been stressed, as has the lack of correlation between the levels of circulating albumin and the extent of fluid accumulation. The total circulating albumin was decreased in every analysis and is presumably the consequence of the failure of the damaged liver parenchyma to elaborate albumin.

The work of Blix in 1941 indicated that a considerable proportion of the lipid material circulating was carried by the β-globulin fraction. Blix, Tiselius and Svensson in 1941 showed further that this and the α-globulin fraction of the circulating proteins were especially rich in cholesterol and allied conjugate structures. Pathological states in which an increase of circulating sterols is known to occur might be expected to show an increase in these fractions, when the method used for the protein analysis is dependent, in part, on detection of refractive changes in the serum. Moreover, Gardner and Gainsborough (1930) have shown that increases in cholesterol may be expected in cases with obstruction to the outflow of bile.

Patients, therefore, in whom there was clinical or laboratory evidence of gross obstruction have been excluded from this series. As a further precaution, where possible, direct analyses have been made of the total cholesterol content of sera actually analysed. All these analyses fell within normal limits. Out of the 18 analyses only two showed an increase in β-globulin beyond normal limits. This suggests that while β-globulin increases may occur they are not the rule in established uncomplicated cases.

Longsworth, Shedlovsky and MacInnes (1939) have shown that in pyrexial patients an increase in the circulating α-globulins may be observed. In the present series special care was taken to collect material from afebrile patients and, in only two analyses was the sum of the globulins above normal limits. Of the remaining 16, 12 fell below the mean of normal levels for the α-globulins.

In contradistinction there was an absolute and persistant increase in the circulating γ-globulin, persisting up to death. It has been asserted, frequently, that the increase in globulin is compensatory in nature. In the present series there is nothing to support this assertion. Thus γ-globulins of the order of 4·7 g. per 100 ml. have been observed within a few hours of death, as well as in patients relatively well compensated. Equally, levels of 1·3 and 1·6 g. per 100 ml. have been observed at the day of death and months before death.

There is an accumulating body of evidence to indicate that γ-globulins in the healthy and the diseased are elaborated in the lymphoid and reticulo-endothelial tissues (Enders, J. F., 1944 ; Dougherty, T. F., Chase, J. H., and White, A., 1944 ; and Martin, N. H., 1947). However, autopsies on patients dying from chronic liver failure, unlike those in acute infective hepatitis, do not

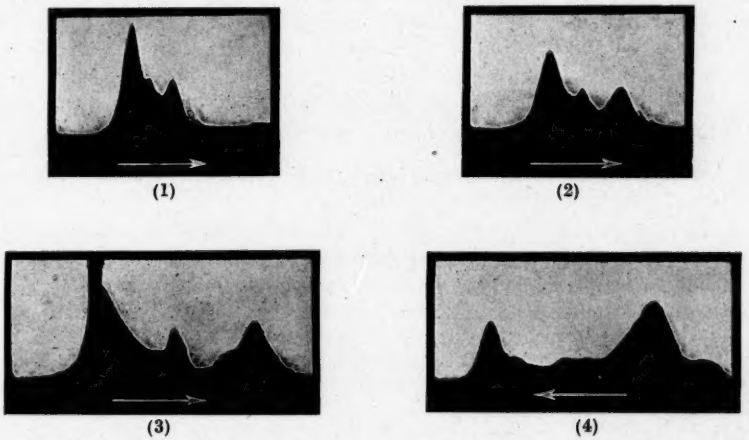

FIG. 1.—Electrophoretic Analysis. Patient No. 6. Arrows indicate the direction of migration.
(1) Descending Limb. 1 hour.
(2) Descending Limb. 2 hours.
(3) Descending Limb. 3 hours.
(4) Ascending Limb. 3 hours.

show widespread hyperplasia of the lymphoid tissue nor of reticulo-endothelial system. Therefore the high levels of circulating γ-globulin observed in all these cases would seem to indicate a failure to dispose of, or modify, γ-globulin in the normal way. The fact that the liver is the primary site of failure in these cases suggests the possibility that it plays a part in the removal of γ-globulin in health. This need not imply that it does more than alter the net charge by conjugation or other means so that it can no longer be identified as a γ-globulin. Nevertheless, such a modification could be of considerable biological significance.

The peculiar behaviour of the γ-globulin in the analysis of Patient 6 deserves special note. Three hours from the start of the electrophoretic run, spontaneous precipitation was observed, localized in a well-defined band in the descending limb of the U-tube only (Fig. 1). All analyses were carried out at a protein concentration of 2 per cent; further concentrations of γ-globulin observed over

the series embrace that in which precipitation was observed. There was no sign of precipitation in the ascending limb. In the ascending limb of the U-tube the γ-globulin concentration is migrating in the presence of the other serum proteins, whereas in the descending limb the γ-globulin concentration is migrating in the presence of the buffer ions only. This would suggest that the associated circulating proteins have a stabilizing effect on globulins in solution.

SUMMARY.

(1) Serial electrophoretic analyses are presented from nine patients suffering from chronic progressive liver failure. In all cases there was a gross increase in the γ-globulin fraction and a marked decrease in the albumin fraction. Spontaneous precipitation of γ-globulin was noted in one patient.

(2) The β-globulin fraction in the majority of the analyses was normal or below normal limits.

(3) The α-globulin fraction in the majority of analyses was below the mean of normal levels.

(4) Ancillary biochemical investigations carried out at the same time as the analyses are listed.

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